

Opiate Receptors
and the
Development of Tolerance to and
Dependence on Morphine

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Jane E. Dum
aus Boston, U.S.A.
Dezember, 1978

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Dissertation der
Fakultät für Biologie
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This paper is dedicated to the thousands of rats and mice which are sacrificed for the sake of science.



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I. ABSTRACT

Various methods were applied to investigate the possibility that changes in opiate-receptor interaction might contribute to the development of tolerance to opiates:

1. In in vitro, comparison of receptor binding of an opiate agonist, etorphine, and antagonist, naloxone, to rat brain homogenate showed no differences between the characteristics of the opiate-receptor in naive and tolerant rats.
2. In in vivo, radioimmunoassay was used to measure the possible effect of naloxone on morphine brain concentrations in naive and tolerant mice. No displacement of morphine from the brain by naloxone could be observed in naive mice or in tolerant mice at increasing intervals after the begin of abrupt withdrawal. Therefore, the suggestion that the affinity of opiate receptors changes during the development of tolerance/dependence based on a displacement of morphine by naloxone, as reported by others, could not be supported.
3. In a second series of in vivo experiments, the displacement of small amounts of radioactive opiate agonist and antagonist from the brain by doses of unlabelled antagonist was used to trace drug-receptor interaction. Differences between the displacement in naive and

tolerant mice did not correlate with the level of tolerance and could be fully explained by the chronic levels of morphine in the brain of the tolerant mice.

4. In behavioral experiments, the ability of naloxone to antagonize morphine analgesia was not different in groups of tolerant rats from that in naive. Thus, no evidence of a change in the affinity constant of the antagonist in tolerant rats was found using this approach.
5. In a second series of behavioral experiments, measurements of the morphine dose-response curves for analgesia in rats showed that, in different groups of highly tolerant animals, the maximum responses produced by the agonist were smaller and the dose-response curves were flatter than in naive animals. The changes in the curves could be best explained by a decrease in efficacy rather than by a decrease in affinity of the drug for the receptor.

II. INTRODUCTION

A. General information about opiates

Morphine is one of the major active components of opium, a derivative of the poppy plant. It is one of a group of natural and synthetic drugs which, through inhibitory action on the central nervous system and probably on the nervous plexus of the wall of the intestine, produces a host of specific effects on man and animals, including analgesia, constipation, respiratory depression, hyperthermia, nausea, sedation and mood changes.

Much pharmacological evidence suggests that opiates produce their effects through binding in the nervous system to distinct receptor sites possessing a specific chemical structure. For example, active opiates all have certain structural characteristics in common. Some opiates produce their effects at very small doses, implying high affinity binding. They are stereospecific, that is, for the most part, only those isomers are active which have a configuration analogous to the D(-)morphine. Also, there exist antagonists, such as naloxone, which produce little, if any, pharmacological effect themselves, but which block the effect of agonists. These antagonists generally differ from agonists only by small chemical changes. This pharmacological evidence has been substantiated by biochemical experiments which have been able to demonstrate high

affinity, stereospecific, opiate binding in the brain and guinea pig intestine (Pert and Snyder, 1973a,b;; Terenius, 1973; Simon et al., 1973; Hitzemann and Loh, 1973; Wong and Horng, 1973).

These receptors have been found to bind endogenous peptides, extracted from the brain (Hughes, 1975; Pasternak et al., 1975; Stewart et al., 1976; Terenius and Wahlström, 1974) and pituitary gland (Cox et al., 1975; Goldstein, 1976; Teschemacher et al., 1975). These peptides themselves produce morphine-like effects and the distribution of some of them in the brain is similar to that of the opiate receptor (Simantov et al., 1976). Therefore, opiates appear to act through direct impingement upon the receptor of endogenous neural substances.

B. General information about opiate tolerance and dependence.

Because of the relative specificity and intensity of opiate activity, the opiates are highly valued for the treatment of pain. Unfortunately, their usefulness is severely limited because tolerance to and dependence on the drugs invariably follow frequent contact with them. Tolerance results in a reduction in the pharmacological responses to the drugs, while physical dependence causes normal functioning to depend on continued drug administration. The interruption of drug treatment or the application of a specific opiate antagonist to dependent humans or animals causes the appearance of a withdrawal syndrome,

consisting of symptoms such as motoric excitement, diarrhea, shivering, hypothermia, as well as depression and irritability in man.

There is convincing evidence that tolerance to and dependence on opiates are closely linked. Dependency develops concurrently with tolerance (Way et al., 1969; Shuster et al., 1963). The two phenomenon follow the same time-course of disappearance (Schulz et al., 1974) and procedures that influence tolerance development also exert an effect on the development of dependence (Way et al., 1968; Loh et al., 1969; Shen et al., 1970; Way et al., 1973). This has lead to the general theory that tolerance and dependence are two expressions of the same mechanism. This concept is also supported by the fact that withdrawal symptoms often appear to be the opposite of acute drug effects. For example, the withdrawal symptoms hypothermia and diarrhea, are the reverse of the acute drug effects, hyperthermia and constipation. Therefore, it is reasoned, tolerance may stem from the establishment of a new equilibrium resulting from compensatory mechanisms allowing the animal to function normally despite drug treatment, while the withdrawal syndrome may be rebound excitation caused by these same mechanisms (Way et al., 1968, 1969; Jaffe and Sharpless, 1968; Martin et al., 1968; Collier, 1966; Goldstein and Goldstein, 1968).

A diversity of mechanisms have been proposed to account for the development of tolerance to opiates. The proposal that the drug effects are reduced due to a decreased concentration of the drug at the site of action because of increased metabolism, elimination or reduced absorption has not been supported by experimental evidence (see Hug, 1972). Neither is the effective concentration of the drug in the brain reduced by combination with antibodies in the tolerant animal, as has been suggested by Cochin and Kornetskey (1964, 1968), since no relationship between immunological mechanisms and tolerance could be shown (Miller et al., 1977) and because large concentrations of free drug, i.e. dialysable, could be found in the brains of such animals (Richter and Goldstein, 1970). Therefore, opiate tolerance must be a kind of cellular or tissue tolerance. That is, changes take place within the nervous system itself which (1) change opiate-receptor interaction, (2) reduce the reaction of the tissue or cell to occupation of the drug receptors or (3) produce compensatory effects exactly cancelling the opiate action. An investigation of the first possibility will be the subject of this paper.

C. Receptor theories of tolerance.

One way in which opiate-receptor interaction might be reduced would be through a reduction in the number of active receptors (Collier, 1968). This hypothesis is based upon

the observation that the number of acetylcholine receptors are reported to increase in response to chronic denervation of skeletal muscles and other peripheral organs, resulting in so called denervation supersensitivity (Axelson and Theseleff, 1959; Theseleff, 1960). Such a mechanism may be important for the modulation of sensitivity since other membrane receptors are sometimes known to respond to a changed concentration of ligand with a change in number (Raff, 1976). This process has been shown, in some cases, to depend on protein synthesis (Galvin et al., 1974) and protein synthesis inhibitors also suppress the development of tolerance to opiates (Cochin and Kornetsky, 1964; Cohen et al., 1965; Cox and Ginsberg, 1969; Smith et al., 1967; Feinberg and Cochin, 1968; Loh et al., 1968; Yamamoto et al., 1967).

A reduction in the affinity of active opiate receptors for opiate agonist has also been proposed as a mechanism of tolerance development. Takemori (1974) has proposed that narcotic analgesics interact with a receptor which is different, but closely associated with that of the antagonist, so that part of the binding sites overlap. In chronically morphinized animals, the agonist receptor may become desensitized, causing tolerance, while the binding of the antagonist may be increased. Snyder (1975) has also suggested a model which pictures a change in the opiate receptor binding during the development of

tolerance. His model stems from the observation that the opiate receptor binding of opiate agonist is reduced by sodium ions, whereas the binding of antagonists is enhanced. Therefore, he reasons, the opiate receptor might exist in two conformations, one sodium dependent, which binds antagonists preferentially, and one not sodium dependent, which binds agonists preferentially. A shift of the opiate receptor towards a sodium dependent conformation would then cause a change in affinity for opiate agonists and antagonists. Such a change in response to chronic opiate treatment could explain tolerance.

A similar hypothesis suggests that an endogenous antagonist might be produced by the body which would reduce the affinity of the receptor for the opiate through some allosteric effect (Ariens, 1966). Such a mechanism has been observed for certain hormone receptors. For example, guanyl nucleotides have been observed to stimulate the dissociation of glucagon and angiotensin from their respective receptors, as well as to profoundly affect the action of peptide hormones and catecholamines (Rodbell et al., 1971; Solomon et al., 1975; Glossman et al., 1974; Birnbaumer et al., 1974). Conceivably, an endogenous antagonist could also block the opiate receptor directly and thus produce tolerance. Such an antagonist has been suggested by Ungar to explain the finding that extracts from tolerant rat brains induce tolerance in naive animals (Ungar et al., 1976; Ungar and Cohen, 1966; Ungar

and Galvin, 1969; Tirri, 1967; Tulunay, 1970), although these findings could not always be repeated (Cox and Ginsberg, 1969; Smith and Takemori, 1968).

Still another possibility which can not be ignored, is that the interaction of opiates with their receptors might be reduced by the opiates themselves, by a kind of autoantagonism (Schmidt and Livingston, 1933). For example, if the opiate receptor only responds when first occupied and fails to respond again until occupied anew, then continuous occupation, resulting from chronically high levels of the drug, would prevent new stimulation. Similarly, if agonists cause a conformational change in the opiate receptor with a long relaxation time, the receptor might fail to respond to new occupation or have a reduced affinity for the drug during this period (Rang and Ritter, 1969; Rang and Ritter, 1970). A drug molecule might also cause an allosteric change in the receptor binding site by occupying a site adjacent to the receptor. Such a reduction in hormone receptor affinity with an increase in hormone concentration, negative cooperativity, is known for insulin (DeMeyts et al., 1973).

D. Intentions of the paper.

This paper will present a group of experiments designed to test the hypothesis that the development of tolerance to narcotics is related to a blockage or change in the number or affinity of the opiate receptor. The emphasis

will be placed on in vivo techniques, although in vitro experiments will also be made. Attention will be given to such variables as degree of tolerance, the effect of withdrawal and the levels of morphine in the brain of tolerant and naive animals. Comparisons between the binding of agonists and antagonists will be made as well as investigations of the interaction between the drugs. In this way it is hoped that a reliable assessment of the hypothesis in question can be made.

III. METHODS: GENERAL CONSIDERATIONS

A. Measurement of high affinity opiate binding in rat brain homogenate.

1. experimental design:

The first experiments were conducted using the method of Pert and Snyder (1973a,b) to compare the receptor binding of opiate agonists and antagonists in rat brain homogenate taken from naive and tolerant/dependent rats. According to this method, brain homogenate is incubated with labelled drug and then quickly filtered and washed with cold buffer. In this way, the high affinity opiate binding is trapped and much of the free and low affinity bound opiate is removed (Pert, 1976). The high affinity bound component of drug left in the tissue is distinguished by performing the same experiment in the presence of increasing concentrations of unlabelled opiates. When this is done, the quantity of bound radioactivity logarithmically decreases down to a plateau, forming a curve which is typical for the displacement of a ligand from a finite number of high affinity sites. The non-displaceable amount probably represents low affinity, non-specific binding due to hydrophobic interactions between the ligands and a wide variety of lipid tissue components (see Höllt and Teschemacher, 1975). The experiments are performed with very low concentrations of

radioactive drug because the proportion of non-specific binding increases with the concentration of labelled opiate used. As a result, drugs with high specific activity are required so that the low concentrations can be detected.

The displaceable quantity of drug seen in these experiments fulfills several criteria for opiate receptor binding: (1) It is stereospecific, that is, only active opiate isomers can displace it at low concentrations. (2) The amount of drug needed to displace the labelled opiate correlates closely with pharmacological potency (Pert and Snyder, 1973a). (3) The binding is primarily found in those tissues sensitive to the drug, i.e. in certain parts of the nervous system.

2. theoretical interpretation:

Any differences between the displacement curves of the naive and tolerant groups would be meaningful. However, a more detailed interpretation of the data is possible when the displaceable binding of opiates is analysed with a Scatchard Plot (Berson and Yalow, 1966; Scatchard, 1949). The theoretical basis for the method is derived from the mass action equation for the ligand-receptor interaction at equilibrium,



which is
$$K_a = \frac{k_a}{k_d} = \frac{[LR]}{[L] \cdot [R]} \quad (2);$$

$$K_d = \frac{k_d}{k_a} = \frac{1}{K_a} \quad (3),$$

where $[L]$ is the concentration of free ligand, in this case free opiate; $[R]$ is the concentration of free receptor; $[LR]$ is the concentration of ligand-receptor complex; k_a is the association rate constant; k_d is the dissociation rate constant; K_a is the equilibrium constant (association constant or affinity constant) in liters/mole ; and K_d is the dissociation constant in moles/liter. Furthermore,

$$[R_t] = [LR] + [R] \quad (4)$$

and
$$[L_t] = [LR] + [L] \quad (5),$$

where $[R_t]$ is the total concentration of receptors and $[L_t]$ is the total concentration of opiate. The number of receptors and their affinity for the drug can be deduced by transforming the binding data as follows:

From equation (2),

$$\frac{[LR]}{[L]} = K_a [R] \quad (6).$$

From equation (4),

$$[R] = [R_t] - [LR] \quad (7).$$

Substituting (7) in (6),

$$\frac{[LR]}{[L]} = K_a ([R_t] - [LR]) \quad (8).$$

A scatchard plot is made by plotting $[LR]/[L]$ (bound drug/free drug) against $[LR]$ (bound drug). According to equation (8), the data then forms a straight line with a slope of $-K_a$ and a horizontal intercept of R_t .

In practice, increasing concentrations of unlabelled drug are incubated in a fixed amount of labelled drug so that a displacement curve can be measured. The quantity of non-specific binding is subtracted. Then the total concentration of displaceable binding of drug divided by the total concentration of free drug in the incubation media is plotted against the total amount of displaceable drug. The presence of different kinds of unlabelled ligands with different affinities would cause a deviation of the plot from a straight line. A deviation in the presence of only a single ligand, however, may be caused by the presence of several binding sites with different affinities, or by the presence of cooperativity (see DeMeyts, 1977; Changeux and Rubin, 1968).

B. Morphine brain concentrations in naive and tolerant mice injected with naloxone as measured with radioimmunoassay (RIA).

1. experimental design:

The next experiment attempts to gain insight into the interaction of morphine and naloxone with the opiate receptor in living naive and tolerant animals, by measuring the total morphine content in the brain of the animals, after acute or chronic morphine treatment, following injection of either naloxone or saline. It is reasoned that a displacement of morphine from the receptor by the antagonist might be accompanied by a drop in total brain concentration of morphine. A larger displacement in tolerant animals, due to an increased affinity of naloxone or decreased affinity of morphine for the receptor, might be reflected in a larger drop in the total brain concentration of morphine.

2. general information about RIA:

The morphine brain concentration is measured with radioimmunoassay (RIA). This presents the advantage that it does not require the injection of radioactive tracer into the animals. The pretreatment morphine is part of the experiment and, therefore, does not present a third variable.

In general, RIA makes use of antibodies which have receptors with high affinity and specificity for the substance measured. Such antibodies are formed by animals inoculated with small amounts of antigen at repeated intervals. Molecules, such as morphine, which are not usually immunogenic, are combined with larger molecules, such as γ -globulin, before inoculation. Injection with Freund's adjuvant also serves to intensify the immune reaction. The antibodies are eventually found in the serum of the animals.

Once the antibodies are recovered from the animals, the binding of a radioactive ligand to the antibody receptors is measured. A standard curve is established by measuring the displacement of this binding by standard amounts of unlabelled substance. When an unknown interacts with the antibody receptor in the same way as the standard and assay conditions are identical, the concentration of the unknown can be estimated by measuring the displacement it causes with reference to the standard curve. The performance of RIA depends upon a quick and dependable method of separating and identifying the specifically bound ligand. In rare instances, antigen-antibody complexes spontaneously precipitate and can be separated by centrifugation. More commonly, the complexes must be precipitated through application of some agent, such as antibody against the antibody or saturated ammonium

sulfate. Frequently, unbound ligand is separated by absorption onto some substance such as charcoal, cellulose or silicates, which can also be removed by centrifugation. (For further details see Yalow and Bearson, 1976).

3. characteristics of RIA:

RIA may be characterized by three variables: sensitivity, precision and specificity. Sensitivity may be defined as the change in tracer binding for a given increment of concentration of unlabelled substance. Therefore, at any concentration of unlabelled substance, the sensitivity of the RIA is the same as the slope of the plot of tracer binding against concentration of unlabelled ligand. Assuming that the tracer and unlabelled ligand compete for one antibody receptor according to the simple law of mass action, Berson and Yalow (1976) were able to show that sensitivity is dependent on the avidity (affinity) of the receptor for the ligands and upon the concentration of unlabelled substance. Following the same reasoning, they were also able to show that sensitivity is optimal when $1/3$ of the tracer is bound and the concentration of unlabelled substance is low. However, in practice, formal analysis of sensitivity can only be approximate, because most antisera are a heterogenous mixture of antibodies having receptors with different avidities and which often deviate from

the simple law of mass action in their interaction with antigens. Sensitivity may also be defined as the smallest concentration of unlabelled substance which can still be detected by the assay (see Ekins and Newman, 1970). This quantity is of special interest, and will be referred to in this paper as the limit of sensitivity. It may be seen that the limit of sensitivity is governed by the concentration of radioactive tracer, because smaller concentrations of unlabelled ligand are able to produce a proportionally greater change in total ligand concentration when only a small amount of tracer is used. Unfortunately, the detection of small amounts of tracer is limited by specific activity. The limit of sensitivity also depends on sensitivity and precision. Precision may be defined as the percentage uncertainty in the estimation of a given concentration of unknown (relative error). Precision generally decreases for lower concentrations of ligand. The final variable which characterizes RIA is specificity. Specificity may be defined as the cross-reactivity of an antiserum with different substances. It can be investigated by determining the relative concentrations of various substances needed to inhibit the binding of the radioactive tracer to the antibody receptors.

C. Opiate high affinity binding in vivo.

1. experimental design:

The object of the next experiment is to study the binding of opiates to the brain of living naive and tolerant animals. This is done by measuring the radioactivity in the brains of animals following injection with radioactive opiates. Low doses of opiate tracer with high affinity for the opiate receptor are applied to maximize the proportion of drug associated with the receptor binding sites (Dobbs, 1968; Cerletti et al., 1974; Höllt et al., 1975). This technique is only possible with the use of drugs having high specific activity, enabling the measurement of low concentrations.

Under these conditions, displaceable opiate concentration in the brain can be visualized when opiate isotopes are injected with increasing doses of unlabelled drug, which causes the radioactivity in the brain to logarithmically decrease down to a plateau (Höllt et al., 1975). The shape of this displacement curve is similar to the displacement curves of saturable receptor binding seen in brain homogenate. The plateau which is reached is reminiscent of the non-displaceable level seen in such experiments. By analogy, the difference between this plateau and the maximum concentration of radioactive ligand is an indicator of the receptor binding.

Unfortunately, this procedure fails when large doses of unlabelled agonists instead of antagonists are applied. In this case, the total concentration of labelled drug remains the same or is, paradoxically, increased (Cerletti et al., 1974; Höllt et al., 1975).

A second and more reliable technique for visualizing receptor binding is that introduced by Höllt and Herz (1976), which compares the concentration of narcotic in the brain without cerebellum to the concentration in the cerebellum. Since the cerebellum has very few opiate receptors (Hiller et al., 1973; Kuhar et al., 1973; Höllt and Herz, 1976; Mulé et al., 1976), the concentration of drug in this structure represents almost entirely free and non-specifically bound substance. As seen in Table 1, increasing doses of unlabelled agonist increase the concentration of labelled drug, as measured by the level of radioactivity, in the cerebellum as well as in the rest of the brain, but the ratio of the concentration of labelled drug in the brain without cerebellum to that in the cerebellum decreases to a plateau. The decrease in the brain/cerebellum ratio may reflect the displacement of labelled opiate from receptor binding sites whereas the increase of concentration in the cerebellum probably represents an increase in concentration in the entire brain, perhaps resulting indirectly from pharmacological

actions of the agonist (Höllt and Herz, 1976; Höllt and Herz, 1978; Manara et al., 1978). It therefore follows that the minimum brain/cerebellum ratio is a measure of the ratio of unspecific binding in the brain to cerebellum.

	unlabelled etorphine (µg/kg)						
	0	3	10	30	100	300	1000
	dpm/g x 10 ⁻³						
brain minus	16.25	18.42	25.24	22.76	20.39	18.25	16.16
cerebellum	± 2.48	± 2.98	± 3.98	± 3.62	± 2.49	± 1.02	± 1.42
cerebellum	6.50	7.84	13.29	13.62	15.36	16.53	14.54
	± 0.82	± 0.61	± 0.98	± 0.71	± 1.20	± 1.47	± 1.28
ratio	2.50	2.35	1.90	1.67	1.33	1.10	1.11

TABLE 1. Radioactivity in the brain (without cerebellum) and in the cerebellum 20 minutes following the i.v. injection of ³H-etorphine (20 µCi/kg or 0.2 µg/kg), together with increasing amounts of unlabelled etorphine. Mean and standard deviation of 12 animals (Table from Höllt and Herz, 1976).

There is much evidence supporting the suggestion that the decrease in the brain/cerebellum concentration ratio reflects a displacement of the labelled narcotic from the opiate-specific receptor. First, the decrease of the ratio down to a plateau points to a saturation of a limited number of binding sites. Secondly, inactive stereoisomers of opiates are unable to effect

the brain/cerebellum ratio. Thirdly, the ratio is decreased by doses of drug which fall within the pharmacologically active range. Finally, the ability of various agonists to reduce the brain/cerebellum ratio correlates with their pharmacological potencies (Höllt and Herz, 1976, 1978).

2. theoretical interpretation:

A more detailed interpretation of the data can be made with a Lineweaver-Burk Plot, which is sometimes applied for the analysis of drug effect (see Goldstein et al., 1974). Instead of effect, the specific drug concentration in the brain ((total drug concentration in the brain) - (total concentration in the cerebellum x minimum brain/cerebellum ratio)) is substituted. The theoretical basis of the method is explained as follows. According to the simple law of mass action, the reaction of a ligand with receptor at equilibrium is represented by the equation

$$\frac{([R_t] - [LR]) \cdot [L]}{[LR]} = K_d \quad (1),$$

where $[R_t]$ is the total concentration of receptors, $[LR]$ the concentration of receptors combined with ligand, $[L]$ the concentration of free ligand and K_d the dissociation constant, which is the reciprocal of the affinity constant, K_a (see also section III A).

From equation (1), it can be shown that

$$\frac{[LR]}{[R_t]} = \frac{[L]}{K_d + [L]} \quad (2).$$

The maximum specific drug concentration is symbolized as $\Delta_{\max}$. Δ is the specific drug concentration at any given dose of unlabelled drug. When the specific drug concentration is proportional to receptor occupation,

$$\frac{\Delta}{\Delta_{\max}} = \frac{[LR]}{[R_t]} \quad (3)$$

and,

$$\Delta = \frac{(\Delta_{\max}) ([L])}{K_d + [L]} \quad (4).$$

When equation (4) is inverted,

$$\frac{1}{\Delta} = \left(\frac{K_d}{\Delta_{\max}} \right) \left(\frac{1}{[L]} \right) + \frac{1}{\Delta_{\max}} \quad (5).$$

This is the equation of a straight line with slope $K_d/\Delta_{\max}$ and a y-intercept of $1/\Delta_{\max}$, when the reciprocals of the specific drug concentrations are plotted against reciprocal concentrations of free ligand. In theory, the dissociation constant may be found by extending the line down to where it intersects the abscissa at $-1/K_d$ and the y-intercept gives $1/\Delta_{\max}$.

Application of the Lineweaver-Burk plot can give some indication if differences between the in vivo displacement curves of naive and tolerant animals

are related to changes in receptor number, to the presence of chronic levels of morphine in the brain, or to a change in affinity of the receptor. This is readily seen if the expression for the Lineweaver-Burk plot in the presence of a chronic morphine concentration is considered. This expression can be derived from the equation for competitive antagonism (see section III D, equation 5), which may be rewritten in the form

$$\frac{[LR]}{[R_t] - [LR]} = \frac{K[L]}{K_m[M] + 1} \quad (6),$$

where $[M]$ is the concentration of pretreatment morphine in the brain; K_m is the association constant of morphine. This expression equals

$$\frac{([R_t] - [LR])[L]}{[LR]} = K_d \left(\frac{[M]}{K_{dm}} + 1 \right) \quad (7),$$

where K_{dm} is the dissociation constant for morphine. From this, the equation for the Lineweaver-Burk plot in the presence of morphine can be derived, as above, for the equation in the absence of pretreatment morphine. This yields the expression,

$$\frac{1}{\Delta} = \left\{ \frac{K_d \left(\frac{[M]}{K_{dm}} + 1 \right)}{\Delta_{max}} \right\} \cdot \frac{1}{[L]} + \frac{1}{\Delta_{max}} \quad (8),$$

which is again the equation for a straight line with y-intercept at $1/\Delta_{max}$. However, the slope of this line is increased by factor $([M]/K_{dm} + 1)$ over that

in the absence of pretreatment morphine. Thus, a chronic concentration of morphine will increase the slope of the Lineweaver-Burk plot without affecting the y-intercept. The extent of the change in the slope caused by a given amount of morphine can be predicted when the relative affinities of the ligand administered and morphine are known.

It should be mentioned that the Lineweaver-Burk plot analysis is based on an idealized model of the data which assumes that equilibrium between opiate and receptor is established, and that the displacement curve is an exact reflection of opiate-receptor interaction. However, in in vivo, the establishment of equilibrium can only be approximate. Moreover, complex pharmacokinetic factors, which are not directly related to opiate-receptor binding, may still influence displacement, despite the use of the cerebellum as an internal standard. The use of the concentration of drug in the cerebellum as an estimate of free drug may also be a source of error since this concentration represents an unspecifically bound quantity as well as a small amount of specifically bound substance, particularly with respect to the antagonist (see section V C.) Thus, it does not strictly reflect the amount of drug free to interact with the receptors. As a result, the affinity constant of the drug will

probably be underestimated. On the other hand, the approximation of the number of specific binding sites should be more reliable. In this light, the Lineweaver-Burk plot may be regarded as a rough tool with which to differentiate some of the factors effecting opiate-receptor interactions in vivo.

D. Measurement of the apparent affinity of an antagonist in living naive and tolerant rats.

1. experimental design:

The object of the next experiment is to investigate the opiate-receptor interaction with an indirect, behavioral approach. The so-called apparent pA_2 value, equal to the negative logarithm of the molar dose of antagonist needed to reduce the potency of an agonist by one-half, will be found. Under certain conditions, to be explained later, the apparent pA_2 is equal to the apparent affinity constant of the antagonist, which is proportional to the logarithm of the affinity constant of the drug. Therefore, a change in the affinity constant of an antagonist during the development of tolerance/dependence should be reflected in a change in the apparent pA_2 value.

2. theoretical interpretation:

The apparent pA_2 method stems from an in vitro method of Clark and Raventos (1939) which estimates the potency of antagonists by measuring their antagonism of agonist action on isolated tissue in a tissue bath. Schild (1947a,b) expanded this concept by defining the pA_x as the negative logarithm of that concentration of antagonist which reduces the effect of an x concentration of agonist down to that of a single

concentration.

The theoretical basis for the method is explained as follows: According to the law of mass action, the combination of agonist, in the absence of antagonist, with the receptor at equilibrium, may be represented according to the following formula :

$$\frac{y}{1 - y} = K_1 A \quad (1),$$

where A is the concentration of agonist at the receptor; K_1 is the association constant of the agonist; y is the proportion of receptors combined with agonist (see also section III A.). In the presence of antagonist, the reaction of agonist with the receptor at equilibrium may be represented as follows:

$$\frac{y'}{1 - y' - z} = K_1 A' \quad (2),$$

where A' is the concentration of agonist at the receptor in the presence of antagonist; y' is the proportion of receptors in combination with agonist; z is the proportion of receptors in combination with antagonist. Likewise, the reaction of antagonist with the receptors at equilibrium is:

$$\frac{z}{1 - y' - z} = K_2 B \quad (3),$$

where B is the concentration of antagonist; K_2 is the affinity constant of the antagonist. Solving for (z),

in equation (2),

$$\begin{aligned}
 K_1 A' (1 - Y') - z k_1 A' &= Y' \\
 \longrightarrow \frac{K_1 A' (1 - Y') - Y'}{K_1 A'} &= z \\
 \longrightarrow z &= (1 - Y') - \frac{Y'}{K_1 A'} \quad (4).
 \end{aligned}$$

Substituting (4) in (3), the equation for simple competitive antagonism can be derived (Gaddum, 1936):

$$\begin{aligned}
 K_2 B \left(\frac{Y'}{K_1 A'} \right) &= (1 - Y') - \frac{Y'}{K_1 A'} \\
 \longrightarrow (K_2 B + 1) \cdot \left(\frac{Y'}{K_1 A'} \right) &= (1 - Y') \\
 \longrightarrow \frac{Y'}{1 - Y'} &= \frac{K_1 A'}{K_2 B + 1} \quad (5).
 \end{aligned}$$

When the same effect is measured in the presence and absence of antagonist, it is assumed that the same fraction of receptors is occupied by the agonist, so that $y = y'$. Then, according to equation (1) and (5),

$$K_1 A = \frac{K_1 A'}{K_2 B + 1} \quad (6)$$

$$\longrightarrow \left(\frac{A}{A'} - 1 \right) = K_2 B \quad (7)$$

Taking the log of both sides,

$$\log \left(\frac{A}{A'} - 1 \right) = \log B + \log K_2 \quad (8).$$

When the ratio of the concentration of agonist in the presence of antagonist to the concentration of agonist in the absence is equal to two (i.e. $A' = 2A$),

$$\log K_2 = -\log B. \quad (9).$$

By definition, $-\log B$ is the pA_2 of the antagonist. As seen in the equation (9), this value is completely independent of the concentration and affinity constant of the agonist and equal to the logarithm of the affinity constant of the antagonist.

Cox and Weinstock (1964) were the first to adapt the pA_2 method to application in vivo. Instead of concentration in a tissue bath, they measured dosages in moles/kg and described the constant they found as the apparent pA_2 . It is apparent because the dose of drug given is not equal to the concentration at the receptor. However, if the dose is proportional to the concentration at the receptor, the apparent pA_2 is proportional to the affinity constant.

The apparent pA_2 is found by graphing the logarithm ((dose agonist in the presence of antagonist/dose agonist in the absence of antagonist) -1) against the negative logarithm of the molar dose of antagonist. As can be seen from equation (8), the data should form a straight line with slope -1 , intersecting with the abscissa at the apparent pA_2 value.

It should be noted that the pA_2 would be affected by the presence of a third substance in the brain, as might be the case in tolerant animals chronically

treated with opiate. When the chronic drug is the same as that which is injected, equation (8) is transformed into

$$\log\left(\frac{A' + \hat{A}}{A + \hat{A}} - 1\right) = \log B + \log K_2 \quad (10),$$

where B is the concentration of antagonist in the presence of the chronic concentration of agonist, $\hat{A}$. The change in the pA_x curve is found when equation (10) is subtracted from equation (8). Then,

$$\begin{aligned} \log B - \log \hat{B} &= \log\left(\frac{A' + \hat{A}}{A + \hat{A}} - 1\right) - \log\left(\frac{A'}{A} - 1\right) \\ \rightarrow \log B - \log \hat{B} &= \log\left(\frac{A + \hat{A}}{A}\right) \quad (11). \end{aligned}$$

Since $\frac{A + \hat{A}}{A}$ is always greater than one, $\log\left(\frac{A + \hat{A}}{A}\right)$ is always positive. It follows that $\log B > \log \hat{B}$ and that $-\log B < -\log \hat{B}$. Therefore, on the pA_x graph, which has a negative logarithmic scale on the abscissa, the pA_x curve, in the presence of an unaccounted for amount of chronic agonist, is moved to the right by the value $\log\left(\frac{A + \hat{A}}{A}\right)$. The shifted line runs parallel to the real line and the difference between the lines is larger when $\hat{A}$ is larger or when A is smaller. Thus, when a chronic level of agonist in the brain is not taken into account, the apparent pA_2 value measured will be exaggerated by $\log\left(\frac{A'}{A} - 1\right)$.

The apparent pA_2 method is only valid as a means for learning about the apparent affinity constant of an antagonist when the competition between the drugs

for the receptor binding site follows the simple law of mass action. That is, when only one molecule of drug combines reversibly with only one receptor, and the binding of the two drugs to the receptors does not effect further binding to the rest of the receptors. When the apparent pA_2 plot has a slope of -1, it suggests that these conditions are fulfilled. A deviation of the slope from this value makes interpretation of the data in terms of antagonist apparent affinity for the receptor impossible. Further evidence for a competitive mechanism of antagonism is presented when the $pA_2 - pA_{10} = 0.95$ (Schild, 1947a, b). The shape of the dose-response curves made in the presence and absence of antagonist may also be used as evidence for a competitive mechanism of antagonists. A competitive antagonist characteristically displaces the dose-response curve of an agonist parallel to the right.

A further requirement for the application of the apparent pA_2 method is that the drugs under investigation be in equilibrium with the receptor when the drug action is measured. This condition is difficult to fulfill in vivo because of the complicated dynamic interplay of absorption and elimination of substances. Timing of testing to coincide with the plateau which appears during the maximum in the time-effect curve comes

closest to equilibrium conditions.

A great advantage in the apparent pA_2 method lies in the fact that it is a null procedure. That is, the technique rests on the identification of drug doses which produce the same effect in the presence and absence of antagonist. Therefore, it is independent of the functional relationship between receptor occupation and effect produced by the agonist. The method only assumes that the production of an equal effect is mediated by the occupation of a constant fraction of receptors in each group (Van Rossum, 1966). Nevertheless, it is important when applying the technique, to screen out factors affecting behavior which are not directly related to receptor occupation by the drug.

E. Measured changes in the dose-response curve of opiates during the development of tolerance and dependence considered in the light of receptor theory.

1. experimental design:

A comparison of the agonist dose-response relationship in naive and tolerant rats is the subject of the next experiment. If the reduction in agonist potency accompanying the development of tolerance is the result of a decline in receptor affinity for the drug, this should be expressed in a parallel shift of the dose-response curve to the right. In contrast, a flattening of the dose-response curve, with a reduction in the maximum effect, would be best explained by a decrease in the efficacy of the drug. Similarly, morphine in the brains of pretreated animals would also cause a flattening of the curve if not taken into consideration. The manner in which these various factors are expected to alter the dose-response curve of the agonist will now be discussed.

2. theoretical basis:

The effect on the dose-response curve of a change in affinity may be predicted from the simple law of mass action. When the agonist interacts with the receptor according to this law, the equilibrium expression for the reaction is

$$\frac{y}{1-y} = K_1 A \quad (1),$$

where A is the concentration of drug at the receptor; K_1 is the association constant of the drug; y is the proportion of occupied receptors (see also section III A.). The relationship between receptor occupation and the log of the drug concentration is, therefore,

$$\frac{y}{K_1(1-y)} = 10^{\log A}.$$

Taking the log of both sides, this becomes

$$\log y - \log K_1 - \log(1-y) = \log A \quad (2).$$

If the affinity is changed, the change in log of the drug concentration needed to occupy a fixed fraction of receptors is then

$$\begin{aligned} \log A - \log A' &= \{ \log y - \log K_1 - \log(1-y) \} - \\ &\quad \{ \log y - \log K'_1 - \log(1-y) \} \\ \rightarrow \log A - \log A' &= \log K'_1 - \log K_1 \quad (3), \end{aligned}$$

where A' is the new concentration of drug and K'_1 is the new affinity constant. Assuming that the occupation of equal numbers of receptors produces equal effects, equation (3) indicates that the difference between the dose-response curves, measured along the log-dose axis, is independent of the dose of drug given, i.e. the curves are parallel. In fact, if the concentrations in the receptor compartments are proportional to the doses given, then the difference between the curves should actually be a measure of

the difference between the logs of the affinity constants.

In this context, it is noted that the ED_{50} of a drug is sometimes taken as a measure of the affinity constant of the drug. The justification for this is also drawn from (1), which, when 50% of the receptors are occupied at ED_{50} , becomes

$$\frac{0.5}{1 - 0.5} = K_1 A \quad \rightarrow A = 1/K_1.$$

Aside from the obvious fact that the concentration of drug within the receptor compartement must be known, there are other problems with the approach. The measurement of ED_{50} is difficult because it depends on the use of a method which can trace the full range of drug action. Even then, the ED_{50} does not necessarily reflect 50% receptor occupation, as spare receptors might exist. In this case, the maximum response would be produced by occupation of less than all receptor sites and less than fifty percent of the receptors would be occupied when 50% of the maximum response is measured.

The dose-response curve of a drug in tolerant animals may also be affected by the level of agonist in the brain due to maintenance on the narcotic. The effect of this chronic level of drug can be derived as follows. According to the simple law of mass action,

the equilibrium of a drug, A, with the receptors in the presence of a second agonist, B, may be symbolized as follows,

$$\frac{y'}{1 - y' - z} = K_1 A' \quad (4),$$

where y' is the proportion of receptors combined with A; A' is the concentration of A at the receptor in the presence of B; z is the proportion of receptors combined with B and K_1 is the affinity constant of A. Similarly, the combination of B with the receptors in the presence of A may be symbolized as

$$\frac{z}{1 - y' - z} = K_2 B \quad (5),$$

where B is the concentration of B and K_2 is the affinity constant of B. Adding equations (4) and (5),

$$\frac{y' + z}{1 - y' - z} = K_1 A' + K_2 B$$

$$\rightarrow \frac{y' + z}{1 - y' - z} = \left[1 + \left(\frac{K_2}{K_1} \right) \frac{B}{A'} \right] K_1 A'$$

$$\rightarrow \log A' = \log (y' + z) - \log (1 - y' - z) - \log \left[1 + \left(\frac{K_2}{K_1} \right) \frac{B}{A'} \right] - \log K_1 \quad (6).$$

If it is assumed that the production of equal effects in the presence of two pure agonists and in the presence of only one is the result of the occupation of the same total fraction of receptors, $y = y' + z$, then, subtracting equation (6) from the log of the equilibrium expression before change in affinity, equation (2), the change in the log dose-

response curve is found,

$$\log A - \log A' = \log \left[1 + \left(\frac{K_2}{K_1} \right) \frac{B}{A'} \right] \quad (7).$$

From equation (7), it can be seen that the difference between the dose-response curves is not constant, but is dependent on A' . The curve is moved to the left and the difference is greatest when A' is small. It follows that the dose-response curve of A is flatter in the presence of B. As would be expected, larger amounts of B in the brain should cause a greater change in the dose-response curve. Finally, the size of the change should be dependent on the ratio of the affinity constant of B to the affinity constant of A. Theoretically, when the ratio K_2 to K_1 and the concentration of B is known, the "true" dose-response curve of A can be estimated from that in the presence of B.

Before the changes in the dose-response curve caused by a reduction in efficacy can be contrasted with those caused by a change in affinity, certain terms must be defined. Efficacy is a proportionality constant which determines the magnitude of a measured response produced when a certain proportion of receptors are occupied by a given ligand. Efficacy may be conceived as being made up of several factors. (1) the number of receptors; (2) the fraction of receptors

which are active; (3) the intensity of the stimulus of the active receptors when occupied by the ligand (intrinsic activity); and (4) the ability of the effector system stimulated by the receptors to express the behavioral response measured (Ariëns et al., 1956; Ariëns, 1964; Riggs, 1963; Rossum and Ariëns, 1962; Rossum, 1966). These factors may vary independently of each other and the product of them is efficacy.

By definition, a reduction in any one or more of the terms making up efficacy causes a reduction in efficacy and, therefore, a reduced magnitude of response to the occupation of a given proportion of receptors. Logically, such a change should cause a flattening of the dose-response curve with a reduction of the maximum response. No change in the ED_{50} should be observed. However, within certain limits, a reduction in efficacy may also cause a parallel shift in the measured dose-response curve to the right. For example, if the efficacy is reduced by a reduction in receptor number, intrinsic activity, or number of active receptors, then the stimulus to the effector system will be decreased, and a smaller response should be produced. In some cases, however, the maximum response recorded is not produced by the occupation of all receptors, because spare receptors exist (see above)

or the entire response curve is not measured by the recording procedure. When this happens, the stimulus to the effector system may be restored by increasing the proportion of receptors occupied. Thus, a parallel shift of the measured dose-response curve would be observed, mimicking the effect that a decrease in affinity would produce. It would be impossible to differentiate if the response was reduced because a smaller portion of receptors are occupied due to a reduced affinity or if the response is reduced, although the same proportion of receptors are occupied, due to a reduced efficacy. This is the disadvantage of this method, which is not a null procedure, like the apparent PA_2 described in section III D, but, rather, is dependent on the functional relationship between receptor occupation and effect produced. This problem can be solved, however, if the reduction in efficacy is large enough so that a reduction in maximum response is visible, and a flattening of the curve can be observed.

IV. METHODS AND MATERIALS

A. Animals and the induction of tolerance

In the experiments with rats, white Sprague-Dawley rats (200-220 gr) were housed six to a box and kept at least three days before begin of experiments. Tolerance was induced by the subcutaneous implantation under narcosis of pellets containing morphine (75 mg as a base) and carrier substances (Gibson and Tingstad, 1970). The schedule of pretreatment is shown in table 2.

In one experiment (estimation of the apparent affinity constant of an antagonist in living naive and tolerant animals), a group of rats which had not been pretreated with morphine pellets was preinjected with one dose of morphine (8 mg/kg, s.c.) six hours before analgesia tests were performed. Rats in abrupt withdrawal received one morphine-containing pellet on the first day and had the pellet removed on the fourth day, six hours before analgesia tests. Analgesia was always estimated sixty minutes after morphine test doses (during peak analgesia). Naloxone was given 20 minutes before analgesia tests.

In the experiments with mice, NMRI mice (25 to 30 gr) were housed at least a week before begin of experiments. Tolerance and dependence was induced by the implantation of a pellet, containing 75 mg morphine base, subcutaneously into the back three days before testing (Way et al., 1969). In the abrupt withdrawal groups, morphine

experi- mental day	PRETREATED GROUPS (TOLERANT)					
	1/4	2/7	3/7	6/10	13/10	24/10
1	1	1	1	1	1	1
2					1	2
3					2	3
4	test	1	2	2	3	3
5						3
6						3
7		test	test	3	3	3
8						3
9					3	3
10				test	test	test

TABLE 2. Pretreatment schedule for the induction of increasing degrees of tolerance in rats. On the experimental days indicated, the different groups received a given number of morphine pellet(s) containing 75 mg/kg morphine each, subcutaneously. Each group is identified with two numbers, the first representing the total sum of morphine pellets implanted, the second the experimental day of testing.

pellets were removed, three days after implantation from the mice at 2, 4, 5, 6 and 8 hours before measurement of tolerance or displacement experiments.

For radioimmunoassay experiments, only half of the brain of each animal was analysed, and two halves (from two mice) were pooled, in order that morphine concentrations could be checked in the remaining halves by another experimenter, using another technique (gas-liquid chromatography).

B. Measurement of high affinity binding in rat brain homogenate.

Within five min. of sacrifice naive and tolerant (6/10 group) rat brains were homogenized with ice cold sodium phosphate buffer, pH 7.4, 50 mM (100 ml/gm), then centrifuged 10 min. at 7500 RPM and the fluid discarded. This was repeated four times with all groups, in order to remove the morphine from the tissue of the pretreated animals. The brain tissue pellets were resuspended in buffer (100 ml/gm) and aliquots of 2 ml were incubated in triplicate at 37°C under constant agitation for five minutes, either alone or with increasing concentrations of unlabelled opiate, and then fifteen minutes longer with the same drug, isotopically labelled. The samples were then immediately suction filtered, under

refrigeration (4°C), through a glass fiber filter and washed twice in quick succession with 5 ml portions of cold (4°C) sodium phosphate buffer. Filters were placed in plastic counting viles with 10 ml scintillation fluid (Demilume). The next day, radioactivity was counted 5 min. at room temperature in a Packard Tricarb Scintillation Spectrometer.

C. Analysis of morphine concentration.

Radioimmunoassay (RIA) was used to measure the morphine concentration in the brains of mice. After acid or organic extraction, a 0.1 ml sample was added to a reaction vial holding 400 μl rabbit morphine antiserum, diluted in 0.05 M sodium phosphate buffer (pH 7.4) and 10% normal rabbit serum. To this, 50 μl ^3H -morphine (2.2 pmoles) was added. After agitation, the reaction mixture was incubated 3 hours at room temperature and then precipitated with cold saturated ammonium sulfate. Following 30 min. incubation (2°C) samples were centrifuged. The supernatant was discarded and the pellet washed with 50% cold saturated ammonium sulfate. The final pellet was dissolved in 1 ml NCS tissue solubilizer (Amersham, Searle, USA) and pipetted into a counting vial containing 10 ml unisolve I (Zinsser, Germany). Radioactivity was counted 5-10 min. using a Packard Tricarb Scintillation Spectrometer. Standard curves were made by adding known amounts of morphine

to antiserum reaction mixture containing samples extracted from brain taken from untreated mice. Recovery was estimated by adding different amounts of morphine to brains before extraction. Maximum serum binding (B_0) for each standard curve - equal to about 1/3 of the ^3H -morphine added - was found by performing the analysis in the absence of unlabelled narcotic; non-specific radioactivity (N) was measured for every experiment by substituting diluted serum from normal rabbits for morphine antiserum.

The antiserum was obtained from a rabbit immunized with carboxymethyl morphine conjugated to bovine serum albumin (Spector and Parker, 1970). The specificity of the antiserum was investigated using the same analysis procedure described above, except that various amounts of inhibitors (dissolved in 0.1 ml) were substituted for the sample. Inhibition curves were linearized by graphing the $(B-N)/(B_0-N) \times 100$ against the $\log$ of the concentration of substance added to the incubation medium on Log-Probit paper, where B_0 = counts bound in the absence of unlabelled morphine, B = counts bound in the presence of inhibition, and N = non-specific binding.

Gas-liquid chromatography, used to affirm RIA results (section V B), to measure chronic morphine in the brains of tolerant rats with pellets (section V B + E) and to

estimate morphine levels in the brains of naive and tolerant rats after morphine injection (section V D), was performed by Gabriele Meyer according to a method which was modified from Dahlström and Paalzow (1975) (see Dum et al., 1977).

D. Extraction of morphine.

Before the performance of RIA, morphine was extracted from the brain with either of two methods. In the first procedure (acid extraction), brains were homogenized in three volumes of 0.01N HCl, centrifuged, and 3 x 0.1 ml of the supernatant were assayed. Recovery was about 92% $\pm$ a standard deviation of about 3% for brain concentrations between 20 and 860 ng/g wet brain. Between 20 and 5 ng/g the recovery was reduced to about 73%, with an average standard deviation of 10%. The second extraction method, which was the same as that used by Shen and Way (personal communication), was adapted from Kupferberg et al. (1964). According to this procedure (organic extraction), the equivalent of one brain was homogenized in 10 ml ice cold n-butanol-chloroform (1:10) with 2 ml ice cold saturated NaHCO₃. The mixture was centrifuged and 8 ml of the organic phase pipetted into a tube containing 1.2 ml 0.01 N H₂SO₄, which was then shaken and centrifuged. 3 x 0.1 ml of the aqueous phase were assayed. Recovery was about 85% $\pm$ a standard deviation of about 10% between 35 and 500 ng/g wet brain. Between

35 and 10 ng/g, the recovery was reduced to only about 60%, with an average standard deviation of 25%. The larger standard deviation in the recovery after organic extraction reduced the precision of the estimates of morphine concentration using this method, especially at lower concentrations. Despite this, this method was used in order to repeat as closely as possible the methods of Shen and Way (1975a,b). The first method, which was simpler and more reliable was employed for comparison.

E. Measurement of in vivo displacement.

In mice, radioactive drugs and saline or unlabelled drugs were injected simultaneously (i.v. dissolved in 100 μ l 0.9% saline) into the mouse tail 20 min. before sacrifice by decapitation. Brains were removed immediately and the brain (without cerebellum) and cerebellum were weighed separately. Radioactivity was measured in the brain parts by scintillation spectrometry following combustion in a Packard Tri-Carb Sample Oxidizer.

In rats (6/10 tolerance group), radioactive etorphine was injected (i.v., dissolved in 200 μ l 0.9% saline) into the rat tail 20 min. and unlabelled morphine (s.c.) 45 min. before sacrifice by decapitation. The rest of the displacement procedure was exactly the same as that in mice.

E. Measurement of analgesia

In rats, analgesia was measured using the slightly modified "vocalization test" (Hoffmeister, 1968), whereby vocalization was induced by an electrical constant current stimulator (rectangular pulses, from 0.01 - 2,000 μ Amps, frequency 50 Hz, duration 10 ms, delivered for 2s) to the root of the tail by means of a bipolar electrode. The percent of animals at any dose for which a stimulus intensity of more than 1200 μ Amps was necessary for the induction of vocalization was taken as percent analgesia.

In mice, analgesia was measured 45 min. after morphine injection i.v. into the mouse tail, during the peak of analgesia, by inducing vocalization with the same electrical stimulator (under the same parameters) as that used for the rats, attached to the mouse by means of an electrode put on the mouse's tail, and on to a copper pipe, wet with saline solution, in which the mouse was placed. Analgesia was taken as the % of animals for which a stimulus intensity of more than 800 μ Amps was necessary for the induction of vocalization. ED_{50} 's were taken from data graphed on log-probit paper.

G. Statistics.

For the analysis of the RIA data, a two dimensional analysis of variance (F test) was performed to see if naloxone had a significant effect under any of the conditions or if an interaction existed between naloxone dose and other factors investigated, i.e. time after pellet removal or time after naloxone injection in the tolerant animals or size of morphine injection in the naive animals, or if a significant difference existed between analytic methods (radioimmunoassay and gas-liquid chromatography) (Senter, 1969).

For the analysis of the in vivo displacement data, ED_{50} 's were calculated from log-probit analysis and a two-dimensional analysis of variance (F test) was performed to see if there was a significant difference between degree of displacement by naloxone, at any of the various doses given, between any of the tolerant/dependent and abrupt withdrawal groups and the naive group; and also if an interaction existed between this displacement and time after pellet removal. ED_{50} 's of naloxone displacement and morphine ED_{50} 's for analgesia with 95% confidence intervals were computed using log-probit analysis (Litchfield and Wilcoxon, 1949).

For the analysis of the data from the measurement of the apparent affinity of an antagonist in living naive

and tolerant animals, significant differences between pA_2 values and slopes of pA_x line and dose-response curves (test for parallelism) were calculated according to Litchfield and Wilcoxon (1949), which method also allowed for the effective use of 0% and 100% values.

H. Drugs.

Morphine HCl (Merck AG, Darmstadt, Germany); Naloxone hydrochloride (Endo Laboratories, Brussels, Belgium); Morphine-3-glucoronide (M-3-g) and dihydromorphine (Research Triangle Institute, Research Triangle Park, North Carolina); 3H -etorphine, 41 Ci/mmol (The Radiochemical Center, Amersham, England); 3H -naloxone, 23.6 Ci/mmol (New England Nuclear Cooperation, Boston); 3H -morphine, 22 Ci/mmol (Radiochemical Center, Amersham, England); Codeine phosphate (Merck AG, Darmstadt, Germany).

V. RESULTS AND DISCUSSION

A. Measurement of high affinity opiate binding in rat brain homogenate.

1. results:

The binding of a radioactive agonist, etorphine, and antagonist, naloxone, in brain homogenate made from the brains of naive and tolerant rats is shown in fig. 1a,b. Increasing concentrations of the respective unlabelled ligands added to the homogenate logarithmically inhibited the binding of the labelled substance down to a plateau. The displacement curves of the drugs are almost identical in naive and tolerant animals.

Scatchard plots derived from these curves are shown in fig. 1c,d. As expected from the displacement curves, the Scatchard plots are nearly the same in the naive and tolerant experiments for both substances. The Scatchard plot for etorphine did not form a straight line. Rather, two slopes may be discerned, reflecting the presence of two binding sites. The site with the highest affinity had a K_a of 2.7×10^9 liters/mole and a maximum capacity of 11 pmoles/gm brain. The site with the lower affinity had a K_a of 3.55×10^8 and a maximum capacity of 16 pmoles/gm wet brain. In contrast, the Scatchard plot for naloxone formed a

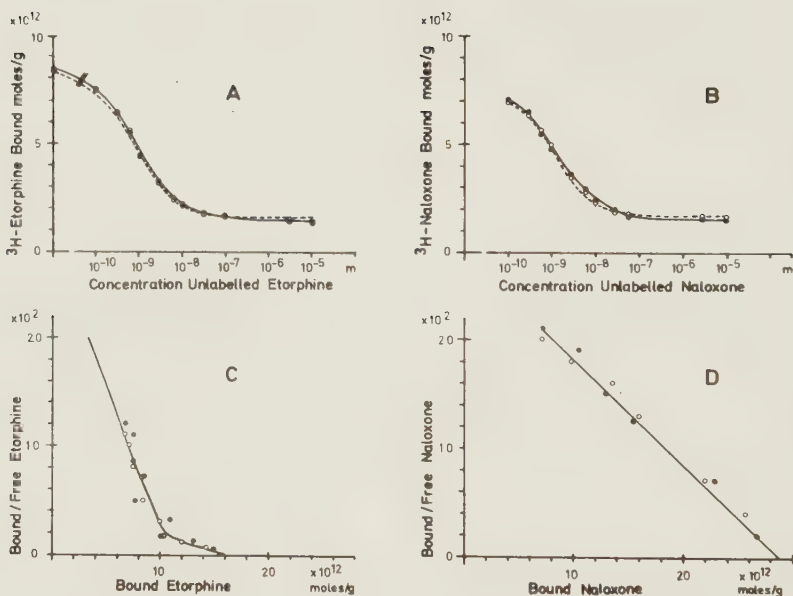


Fig. 1: Inhibition of specific opiate binding of ^3H -etorphine (A) and ^3H -naloxone (B) by the respective unlabelled ligands in brain homogenate of naive (●—) and tolerant (○---) rats. Scatchard plots for the binding data in (A) and (B) are shown in (C) and (D), respectively. The etorphine plot indicates two binding sites, a high affinity site $K_a = 2.7 \times 10^9$ liters/mole, with a capacity of 11 pmoles/gm brain, and a low affinity site, $K_a = 3.55 \times 10^8$ liter/mole, with a capacity of 16 pmoles/gm wet brain. The naloxone plot indicates only one binding site, $K_a = 9.86 \times 10^8$ liters/mole, with a capacity of 29 pmoles/gm wet brain.

single straight line, indicating only one binding site for the antagonist with K_a of 9.86×10^8 liters/mole and a maximum capacity of 29 pmoles/gm wet brain.

2. discussion:

The binding of both the agonist and antagonist was essentially identical in brain homogenate from naive and tolerant rats. This agrees with other in vitro studies (Klee and Streaty, 1974; Bonnet et al., 1976). There appeared to be two binding sites for the agonist. This substantiates the findings of Simon et al. (1975), who also found two binding sites for etorphine in the presence of sodium ions. The affinities which Simon reports for these sites, 1.43×10^9 and 2.5×10^8 , are also very similar to those found in this study, 2.7×10^9 and 3.55×10^8 . Pasternak and Snyder (1975) also detected high and low affinity binding sites for an opiate agonist in the presence of sodium. For naloxone, in contrast, only one binding site could be seen in this study. Interestingly, the affinity of the high affinity binding site of Pasternak and Snyder, 2.5×10^9 , was similar to that found in this investigation, 9.86×10^8 . Birdsall and Hulme (1976) and Simon et al. (1975) also measured only one binding site for the antagonist in the presence of sodium. Both authors

found that the total number of receptors for agonists and antagonists remained about the same under sodium. This is in line with the results of this paper, since the total sum of binding sites for the agonist, 27 pmoles/gm brain, is very close to that measured for the antagonist, 29 pmoles/gm brain.

B. Morphine brain concentrations in naive and tolerant mice injected with naloxone or saline as measured with radioimmunoassay (RIA).

The previous experiment was unable to find changes in the opiate receptor during the development of tolerance through the measurement of opiate binding in vitro. However, the possibility can not be ignored that changes taking place in the receptor of the living tolerant animal might be disturbed by the in vitro procedure. Therefore, the following experiments center their attention upon the concentration of the opiate agonist, morphine, in the brains of naive and tolerant animals in vivo, in order to compare any possible effects made by an antagonist, naloxone, upon morphine concentration in the brains of naive and tolerant mice, using radioimmunoassay.

1. characteristics of radioimmunoassay:

A typical standard curve, made with incubation medium containing HCl from acid extraction, is presented in fig. 2. One pmole of unlabelled morphine in the anti-serum assay tube (2 pmoles/ml concentration) caused about a 20% inhibition of ^3H -morphine binding. The binding of ^3H -morphine by antiserum was reduced if H_2SO_4 from the organic extraction was added to the incubation medium, instead of the HCl. Nevertheless,

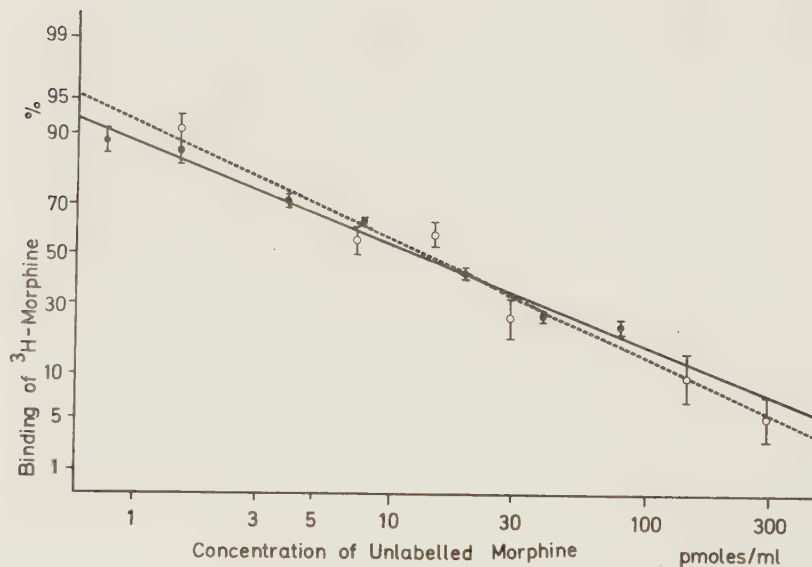


Fig. 2: Inhibition of the specific binding of ^3H -morphine to antibody by increasing concentrations of unlabelled morphine in incubation media containing HCl from acid extraction (●—) and H_2SO_4 from organic extraction (○---) of mice brains. The slopes of the two curves (sensitivity) are very similar, although the concentration of antibody needed after organic was 30% greater than after acid extraction.

a similar standard curve could still be found when a 30% more concentrated solution of antiserum was used (see fig. 2). The standard deviation of an estimate of a single brain concentration (relative precision) was higher after organic (10%) than after acid (3%) extraction, because of higher variation in recovery (see section IV D.). Therefore, the limit of sensitivity for the detection of a morphine concentration in a single brain was higher for the organic (10 ng/g wet brain) than for the acid (6 ng/g wet brain) extraction, although the slopes of the two standard curves (sensitivity) were about the same for the two methods.

The results of tests of the specificity of the antiserum are presented in fig. 3. Of the substances tested, the antiserum had only a minimal cross-reactivity with normorphine and morphine-3-glucuronide, and almost none with naloxone. However, it bound codeine somewhat better than morphine itself.

2. morphine brain levels in mice:

The first experiments were performed with naive mice. Doses of morphine were injected into previously untreated mice in combination with naloxone administration or saline. The increasing doses of morphine

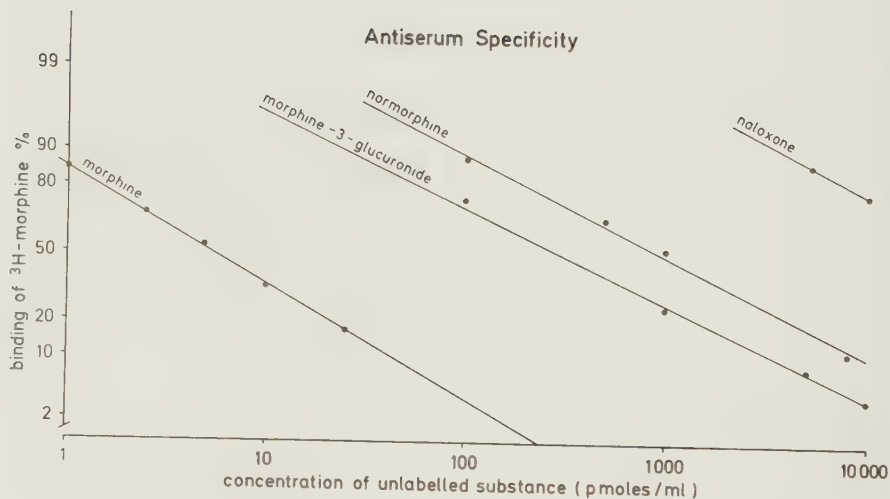


Fig. 3: Comparison of the inhibition of specific ^3H -morphine binding to antibody by different unlabelled substances and morphine. Codeine binds somewhat better to the antibody than morphine.

produced increasing morphine brain levels which were not significantly ($p > 0.05$) effected by naloxone (see fig. 4), as measured by RIA (acid extraction).

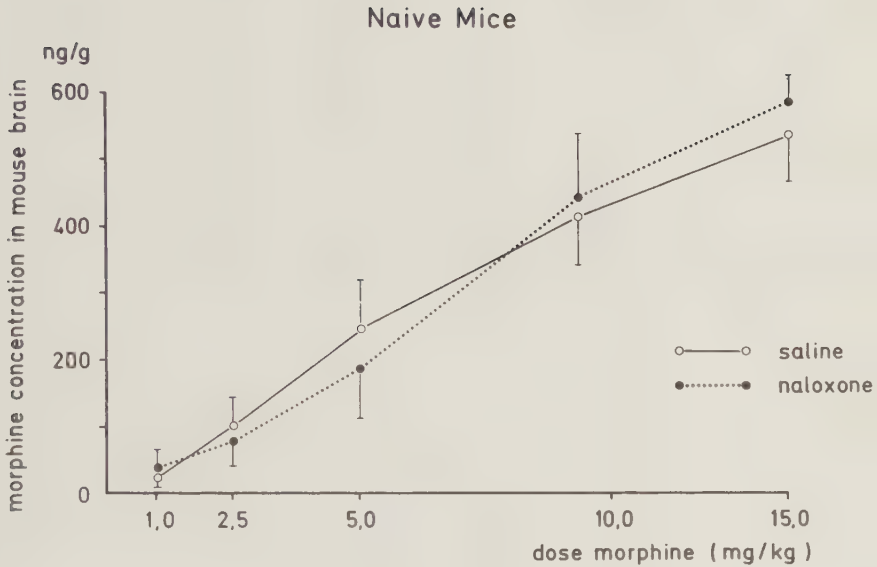


Fig. 4: Concentration of morphine in the brains of naive mice injected with morphine (i.v.) 45 min. before sacrifice and physiological saline or naloxone (10 mg/kg i.p.) 15 min. before sacrifice. Values are the means of 10-12 mice, with standard deviations. There is no significant difference ($p > 0.05$) between any of the saline and naloxone groups.

3. morphine brain levels in tolerant animals and during withdrawal (after pellet removal):

The results of the tolerant experiments, using the organic extraction procedure, are presented in fig.

5a. After removal of the morphine pretreatment pellet, a first order elimination of morphine from the brain was observed ($t_{1/2}$ approximately 2 hours) and no significant effect ($p > 0.05$) of naloxone on morphine concentration could be seen. Concentrations were reduced from about 300 ng/g down to roughly 18 ng/g at 8 hours after pellet removal. The results of this experiment using gas-liquid chromatography to measure the morphine content in the brain were nearly identical (fig. 5b). A repetition of the experiment using RIA in combination with the simpler acid extraction, also produced the same results.

In all of the above experiments, morphine levels were measured 15 minutes after naloxone injection. Therefore, another experiment, performed with RIA (organic extraction) and GLC, was conducted to analyse morphine levels at different times following antagonist administration. This was done 5 hours after pellet removal in tolerant mice. As seen in fig. 6, morphine concentration at this time varied around 50 ng/g brain and failed to decrease significantly at any of the times

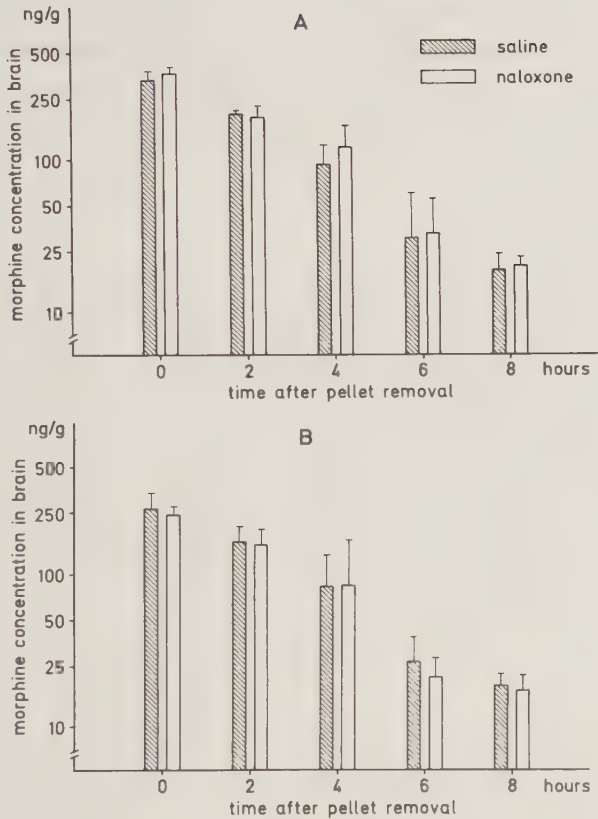


Fig. 5: Morphine brain levels in tolerant mice at different times following morphine pellet removal. Levels measured with RIA (A) and GLC (B). Animals received either naloxone (10 mg/kg i.p.) or physiological saline 15 minutes before sacrifice. Each column is the mean of 10-12 mice with standard deviation. There is no significant difference ($p > 0.05$) between saline and naloxone groups.

tested after naloxone injection. On the contrary, a slight but consistent tendency was seen towards an increase in morphine concentration in the brains of naloxone-injected mice. The same results were found in parallel experiments with gas-liquid chromatography (fig. 6).

4. discussion:

No significant ($p > 0.05$) change in morphine brain levels in either naive or tolerant mice could be found in response to a large dose of naloxone (10 mg/kg). This is in contrast to the findings of Shen and Way (1975 a,b), who reported a dramatic decrease in morphine levels in tolerant mice following naloxone administration. The reason for this discrepancy is not known since our experimental procedure - including range of initial morphine concentrations, treatment schedules, experimental animal and method of extraction and analysis - was very similar to theirs. The use of another form of extraction, which improved the precision and the limit of sensitivity of the assay, was also unable to make a naloxone effect on morphine concentration visible. The specificity of the antiserum used in this investigation is very similar to that used by Shen and Way (1975a,b). Therefore, it is unlikely that antibody specificity can account for the

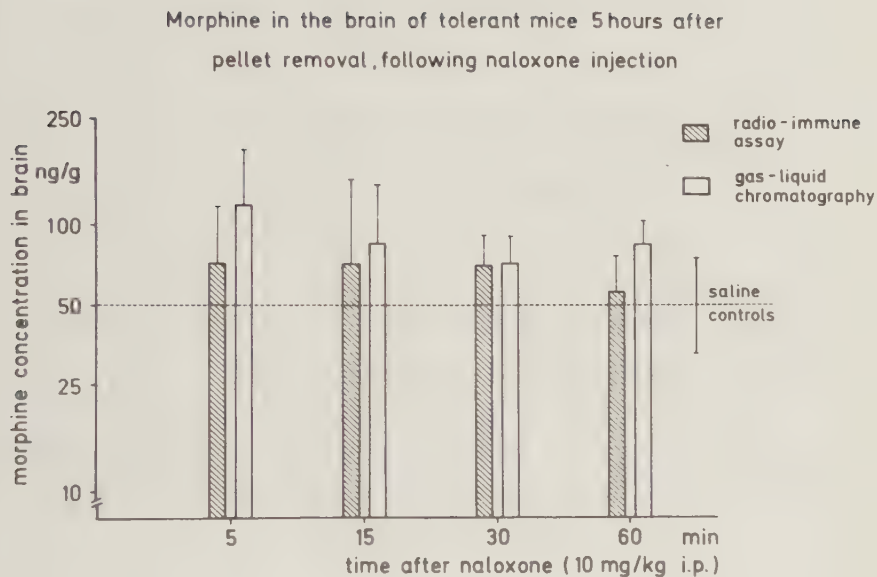


Fig. 6: Morphine brain concentration in tolerant mice 5 hours after morphine pellet removal. Animals received naloxone (10 mg/kg i.p.) or saline at various times before sacrifice. Morphine levels were measured with either RIA or GLC. Saline controls, represented by the horizontal line, were combined (30 mice). Each column is the mean of 10-12 mice + standard deviation. No significant difference ($p > 0.05$) could be seen between groups.

discrepancy between laboratories. Furthermore, the concentrations measured in this experiment agree, in general, with those found by other investigators in tolerant mice (Catlin et al., 1977a, and Patrick et al., 1975). The same experiment done in this laboratory using gas-liquid chromatography, instead of radioimmunoassay, also agreed closely with these results. It must be said, however, that a rather high variability of the morphine brain concentration in tolerant mice would make the detection of a small change in morphine levels difficult, although effects as large as those reported by Shen and Way (1975a,b) would definitely have been seen. Catlin et al. (1977a) was also unable to find a displacement of morphine from the brains of tolerant mice.

The fact that no displacement of morphine from the brain by naloxone could be observed in our experiment does not mean that morphine is not displaced from the receptor. Bound opiate, in relationship to the total concentration, is probably too low to be detectable, reflecting the relatively low affinity of morphine for opiate receptors under in vivo conditions. This assumption is supported by studies (Höllt et al., 1975) in which in vivo displacement from brain by antagonists of the potent agonist, etorphine, could

be detected, in contrast to substances having a lower receptor affinity, such as morphine and dihydromorphine (see Manara et al., 1978).

Measurement of the decay of morphine concentration in the brains of tolerant/dependent mice when the morphine slow-releasing depot pellet was removed (see methods and materials), showed that there is a first-order elimination which reduces the morphine brain concentration from 320 ng/g down to 18 ng/g within 8 hours. Therefore, within the range of concentrations measured, there is no indication that morphine becomes permanently fixed to nervous tissue in tolerant/dependent mice.

C. Opiate high affinity binding in vivo.

Because the previous experiment was unable to register the displacement of morphine from the brain using radio-immunoassay, the following experiment uses highly labelled radioactive isotopes in order to trace opiate displacement, so that a comparison of the effect of naloxone on the concentration of an opiate agonist and antagonist in the brains of naive and tolerant animals can be made. In a preliminary experiment, ^3H -morphine was injected (2 μCuries /mouse i.v.), simultaneously with saline or a large dose of naloxone (10 mg/kg i.p.) 20 minutes before sacrifice. No effect on the brain concentration of morphine in either tolerant or naive mice could be observed under the influence of the antagonist. Therefore, further displacement experiments were performed using ^3H -etorphine and ^3H -naloxone, which are known to have higher affinities for the specific opiate receptor. The results of these experiments were also correlated with tolerance.

1. displacement experiments with ^3H -etorphine:

The experiments with the agonist, etorphine, are represented in fig. 7. ^3H -etorphine was injected simultaneously with saline or increasing doses of unlabelled naloxone and the concentrations of labelled drug were determined separately in the brain without cerebellum (brain) and cerebellum 20 minutes later. In all experiments, naloxone drastically reduced the concentration

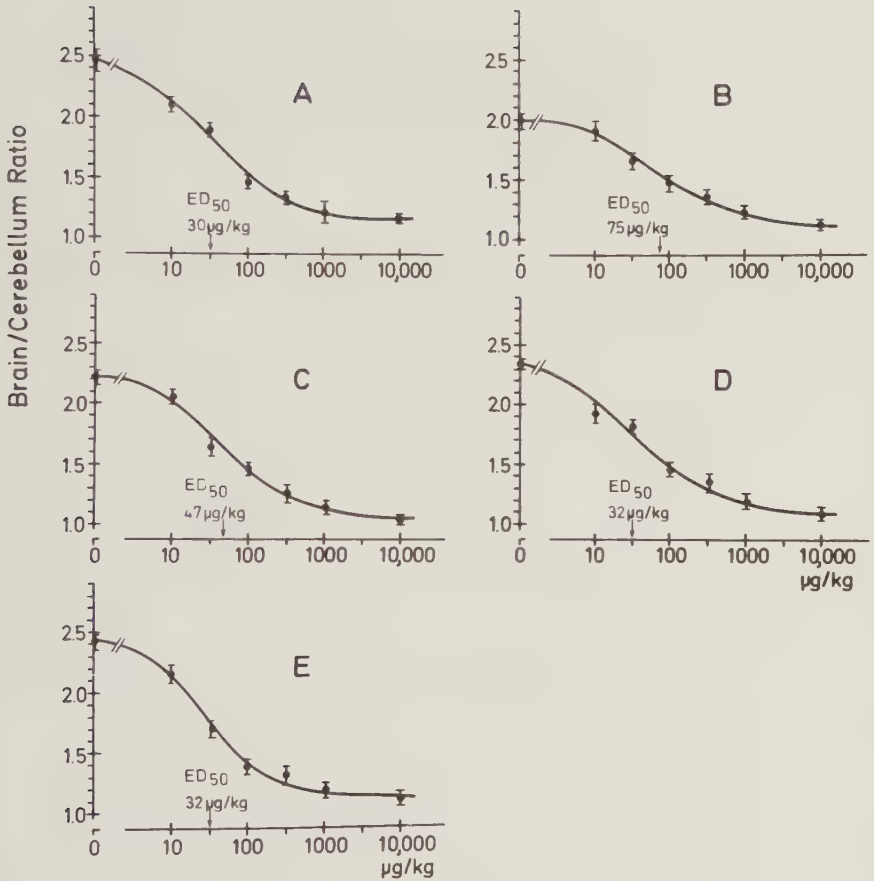


Fig. 7: The reduction of the ratio of the concentration of ^3H -etorphine in the brain (without cerebellum) to concentration in the cerebellum (brain/cerebellum ratio) by increasing doses of unlabelled naloxone in naive mice (A) and in mice made tolerant by the s.c. implantation of a morphine pellet three days before the experiment: with pellet (B) and 4 (C), 8 (D), or 16 (E) hours after pellet removal (in abrupt withdrawal). Etorphine (0.2 $\mu\text{g/kg}$) and naloxone injected (i.v.) simultaneously 20 min. before sacrifice.

Standard error indicated. 70 mice in each curve. ED₅₀ naloxone illustrated. Curves after eight (D) and 16 (E) hours of withdrawal are no longer significantly different from that of the naive mice (A) ($p > 0.05$).

of ³H-etorphine in the brain, without significantly affecting the concentration of the labelled drug in the cerebellum, which averaged for the various groups about 0.064±0.01 pmoles/gm. Doses of naloxone larger than 1000 µg/kg reduced the brain concentration of ³H-etorphine down to a plateau. At this point, the ratio of the concentration of ³H-etorphine in the brain to the concentration in the cerebellum was about 1.1. The displacement curve of tolerant animals was much shallower than that of the naive animals (significant $p < 0.01$). In the naive mice injected with etorphine and saline, an etorphine concentration of 0.157 pmoles/gm was found in the brain, causing a brain/cerebellum ratio of 2.48. The dose of naloxone which reduced the brain/cerebellum ratio half-way down to minimum (ED₅₀ naloxone) was 33 µg/kg. In tolerant mice, the displacement curve was significantly different from that in the naive mice ($p < 0.01$). The concentration of ³H-etorphine in the brain after injection of etorphine and saline was only 0.104 pmoles/gm, which is reflected in a lower brain/cerebellum ratio, 1.9. The ED₅₀ naloxone was increased to 75 µg/kg. When these mice were brought into abrupt

withdrawal, the difference between the displacement curves of the naive and pretreated mice began to disappear. After 4 hours the displacement curve was still significantly different from that of the naive mice ($p < 0.01$), although the brain concentration of ^3H -etorphine in the absence of naloxone had increased to 0.148 pmoles/gm, resulting in a larger brain/cerebellum ratio, 2.21, and the ED_{50} had declined to about 47 $\mu\text{g/kg}$. Eight and sixteen hours after being brought into abrupt withdrawal, there was no longer a significant difference ($p > 0.05$) between the displacement curves of the morphine-pretreated and naive mice.

2. displacement experiments with ^3H -naloxone:

The results of the same experiment with the antagonist, naloxone, were similar to those seen with the agonist (see fig. 8). The labelled naloxone (0.34 $\mu\text{g/kg}$) was injected simultaneously with saline or increasing doses of unlabelled naloxone and the concentrations of the labelled antagonist in the brain and cerebellum 20 minutes later were determined separately. ^3H -naloxone was displaced from the brain by increasing doses of unlabelled naloxone. A small displacement, about 9%, was also seen in the cerebellum in the naive group. The brain/cerebellum ratio was reduced in both naive and tolerant mice to 1.3 by a naloxone

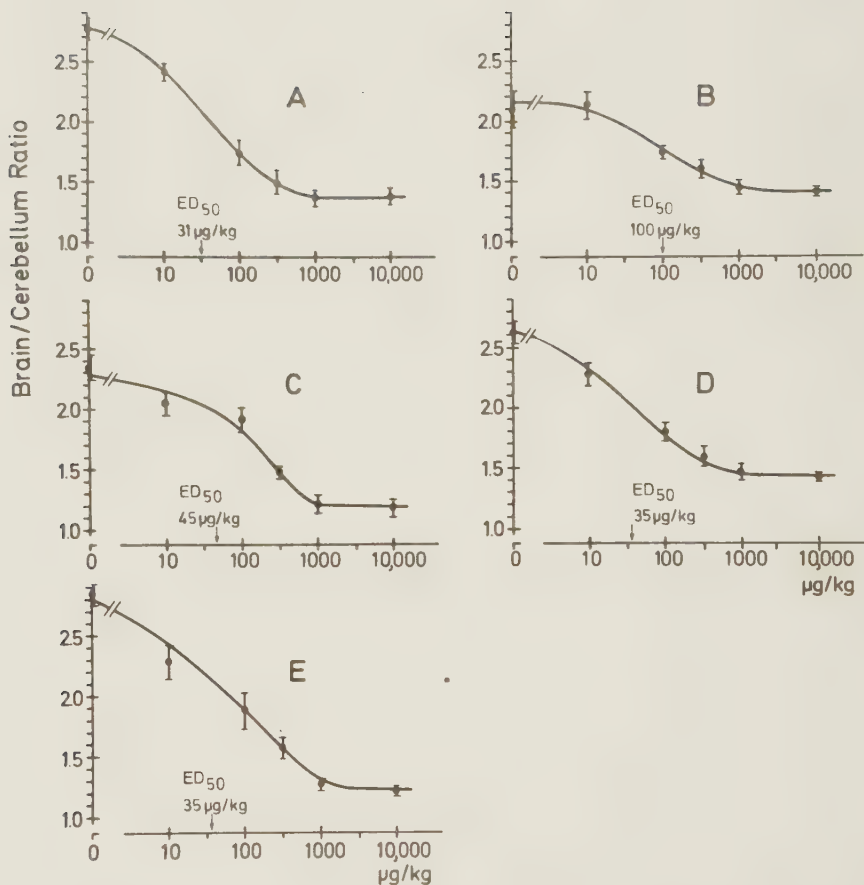


Fig. 8: The reduction of the ratio of the concentration of ^3H -naloxone in the brain (without cerebellum) to concentration in the cerebellum (brain/cerebellum ratio) by increasing doses of unlabelled naloxone in naive mice (A) and in mice made tolerant by the s.c. implantation of a morphine pellet three days before experiment: with pellet (B) and 4 (C), 8 (D) or 16 (E) hours after pellet removal (in abrupt withdrawal). ^3H -naloxone

(0.34 $\mu\text{g/kg}$) and unlabelled naloxone injected i.v. simultaneously 20 min. before sacrifice. S.E. indicated. 50 mice to each curve. ED_{50} unlabelled naloxone illustrated. After 8 and 16 hours of withdrawal (D) the displacement curves are no longer significantly different from that of the naive mice (A) ($p > 0.05$).

dose between 300 and 1000 $\mu\text{g/kg}$, and could not be further decreased by larger doses of unlabelled antagonist.

The displacement curve for ^3H -naloxone of the naive mice was considerably steeper than that of the mice made tolerant by morphine pretreatment (significant $p < 0.01$). In naive mice injected with ^3H -naloxone and saline, the ^3H -naloxone concentration in the brain (0.4 pmoles/gm) was higher than that in the tolerant mice (0.3 pmoles/gm), causing a higher brain/cerebellum ratio (2.78) in the naive than in the tolerant animals (2.1). The ED_{50} of naloxone (31 $\mu\text{g/kg}$) was also much smaller in the naive than in the tolerant group (100 $\mu\text{g/kg}$). When the morphine-pretreatment group was brought into abrupt withdrawal, the difference between them and the naive group began to disappear. As in the agonist experiment, the displacement curve of the pretreated animals four hours after being brought into abrupt withdrawal was still significantly different ($p < 0.01$) from that of the naive, although

the brain/cerebellum ratio after injection with saline was increased and the ED_{50} had declined. Eight and sixteen hours after pellet removal, there was no longer a significant difference between the displacement curves of the pretreatment and naive animals.

Lineweaver-Burk plots for the naloxone data are shown in fig. 9. (Plots can not be made for the etorphine data because etorphine was inhibited by naloxone instead of unlabelled etorphine). The slope of the plot for the tolerant mice (with pellet) is almost twice that of the plot for the naive animals. As a result, the estimate of the affinity constant for the tolerant animals, 2.36×10^7 , is smaller than that for the naive, 5.66×10^7 . In contrast, the estimate of the number of binding sites remains around 20 pmoles/gm brain for all groups. After the tolerant animals are brought into abrupt withdrawal by removal of the morphine pellet, the difference between the plots of the naive and pretreated animals disappears.

3. levels of tolerance in morphine pretreated mice before and during abrupt withdrawal:

Morphine dose-response curves for analgesia were measured in the naive and tolerant groups having an implanted morphine pellet and after being brought into abrupt withdrawal (following pellet removal) to see

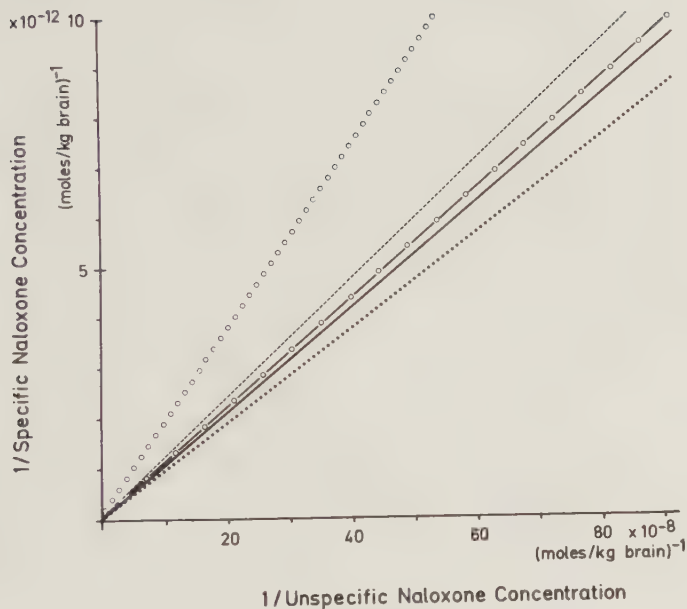


Fig. 9: Lineweaver-Burk plots of the displacement of ^3H -naloxone from the brain by increasing doses of unlabelled naloxone in naive mice (—), tolerant mice with pellet (•••••), and in mice in abrupt withdrawal: 4 hrs (-----), 8 hrs. (•—•—•) and 16 hrs. (•••••) after morphine pellet removal. The plot of the tolerant animals with pellet is twice as steep as that of the naive. The number of binding sites (about 20 pmoles/gm brain) remains about the same in all groups.

if the changes in the displacement of the labelled agonist and antagonist by unlabelled naloxone could be related to changes in tolerance.

The morphine ED_{50} for analgesia curves for the various groups are represented in Table 3. The dose-response curve of the pelleted tolerant mice was adjusted to correct for the amount of morphine in the

GROUP	NAIVE	MORPHINE PRETREATED		
		PELLET LEFT IN	8 HOURS WITHDRAWAL	16 HOURS WITHDRAWAL
ED_{50} MORPHINE (mg/kg)	5.2 { 8.8 3.1 Δ	71 { 135 36 *	78 { 133 46 *	22 { 44 11 Δ *

Table 3: Morphine ED_{50} analgesia with 95% confidence interval in naive mice and in mice made tolerant by the s.c. implantation of a morphine pellet three days before the experiment: with pellet left in and 8 or 16 hours after the pellet removal (withdrawal). 40 mice in each group. * significantly different from naive mice ($p < 0.05$). Δ significantly different from tolerant mice with pellet ($p < 0.05$). The ED_{50} of the "pellet left in" group comes from dose-response curve adjusted to compensate for the pretreatment level of morphine ("pellet morphine") in the brain (see results).

brain by adding the dose of morphine necessary to produce this amount to the dose actually given, so

that the naive and different tolerant curves could be directly compared. The dose morphine needed to produce analgesia in 50% of the naive mice was about 5.2 mg/kg. After the mice were made tolerant by s.c. implantation of a morphine pellet, this dose was increased to 71 mg/kg. The mice in abrupt withdrawal, eight hours after the morphine pellet was removed, were still as tolerant as the pelleted animals. Sixteen hours after pellet removal, the morphine ED_{50} for analgesia was still about four times greater than that found in naive mice.

4. discussion:

The administration of increasing doses of the narcotic antagonist, naloxone, lowered the concentration of the agonist, 3H -etorphine, and of the antagonist, 3H -naloxone, in the brain (without cerebellum) of mice. A small displacement of 3H -naloxone from the cerebellum in the naive group of mice probably reflects a small population of antagonist receptors in this region, as reported by others (see Höllt and Wüster, 1978). In all animals, a Lineweaver-Burk plot analysis of the 3H -naloxone data estimated the number of binding sites in the brain to be about 20 pmoles/gm wet brain, which is in good agreement with the in vitro data (see section V A.). In the naive group, this analysis estimated the affinity of the receptor for naloxone to be

5.65×10^7 . Although this is lower than the estimate made in the in vitro experiment, it is close to the affinity of the "low affinity" binding site measured in vitro by Pasternak and Snyder (1975) (see section VI for further discussion). A preliminary experiment using ^3H -morphine was unable to measure a displacement of morphine with naloxone. This finding is in line with the previous experiment, which could not measure a displacement from the brain of morphine by naloxone using radioimmunoassay, probably because of the relatively low affinity of the agonist (see discussion of section V B). It should be noted that since this method is apparently unable to trace the displacement of substances which have a relatively low affinity for the binding sites, the presence of such binding sites for ^3H -etorphine or ^3H -naloxone, in addition to the high affinity sites measured, would also be undetected.

A comparison of the brain/cerebellum ratios of the highly labelled agonist and antagonist and the reduction of this ratio by naloxone in naive and tolerant mice before and during withdrawal revealed some differences. In the brains of the tolerant animals injected with ^3H -etorphine or ^3H -naloxone, the brain/cerebellum ratios were lower than in the naive group and the ED_{50} s of naloxone to reduce these ratios

were higher. The increase in the ED_{50} of naloxone and the decrease in the concentration of 3H -naloxone in the brains of the tolerant and dependent animals are counter to behavioral observations, which tend to show an increase in effectiveness of the antagonist in tolerant and dependent animals (Way et al., 1969).

Construction of Lineweaver-Burk plots of 3H -naloxone displacement enables the changes in the displacement data to be more closely analysed. Since the y-intercept of the plots of the tolerant mice remain unaltered, there is no indication of a change in the number of opiate receptors. The increase in slope from about 1×10^3 to about 2×10^3 might be related to either a reduction in affinity of the receptor or to competition of the chronic morphine in the brains of the pretreated animals for the binding sites. A given amount of morphine should theoretically increase the slope of the Lineweaver-Burk plot by an amount equal to the concentration of morphine times the relative affinity of morphine to that of the injected ligand, all divided by the number of binding sites (see section III C). The morphine concentration in the tolerant animals is about 10^{-6} moles/liter (see section V B). The relative affinity, morphine: naloxone, is about 1:48 (Creese and Snyder, 1976). The number of binding sites is about 20×10^{-12} moles/

gm wet brain. This data predicts an increase in the slope of the plot of about 1×10^3 . Therefore, the morphine concentration is able to completely account for the differences between the plots of the naloxone data of the naive and tolerant animals. Furthermore, the differences between the displacement curves of the agonist and antagonist disappear as morphine leaves the brain during abrupt withdrawal, although tolerance is still high. No significant change in the displacement curves of the tolerant/dependent mice could be observed as tolerance began to decline between eight and sixteen hours after the beginning of abrupt withdrawal. Thus, the differences between the displacement curves of the naive and tolerant animals does not seem to be related to tolerance, but, rather to the morphine in the brain, which also competes for receptor binding sites.

Other researchers, studying the absorption of opiates in tolerant/dependent animals, have also found reduced amounts of opiates in the brains of tolerant as compared to naive animals (Mulé and Woods, 1962; Goldstein et al., 1973; Misra et al., 1973). These results would appear to agree with ours in tolerant/dependent animals not in abrupt withdrawal; however, they can not be understood in the same way. For the most part, these experiments were performed with drugs having relatively

low affinity for opiate receptors. As a result, the concentrations of the drugs measured in the brains could only have been associated to a small degree with opiate-specific binding sites and changes in these concentrations would probably not reflect opiate-receptor interactions. Another experiment, performed with the high affinity agonist, etorphine, also found reduced absorption into the brain of opiate in tolerant animals, although the level of pretreatment drug in the brain was very low (Luini and Manara, 1977). These puzzling results may be explained by the fact that all of these experiments made use of doses of agonist which were effective in the naive animals. As previously reported (see Höllt and Herz, 1978), effective doses of agonists can cause an unspecific increase in brain levels of small amounts of injected drug. Thus, the decreased concentration of drugs in the brains of tolerant/dependent animals probably did not result from changes in opiate-receptor interactions, but rather from pharmacokinetic changes secondary to agonistic effects of opiates in non-tolerant animals. This suggestion is supported by other in vivo experiments, in tolerant animals having little pretreatment drug in the brain, which, by reducing the influence of unspecific factors by studying the binding of ^3H -etorphine in brain tissue fractions enriched in opiate receptors, found no change in

the quantity of drug associated with stereospecific opiate binding sites (Manara et al., 1978; Mulé et al., 1976). Thus, these last experiments may be compared to ours in tolerant/dependent mice after eight hours of abrupt withdrawal, which by using high doses of antagonist, instead of agonist, and by computing the brain/cerebellum ratio, also reduced unspecific pharmacokinetic factors and could find no change in opiate-receptor interaction.

Another interesting finding of this investigation is that the continuous occupation of the receptors by the agonist does not appear to be directly responsible for the maintenance of tolerance. Instead, a time-dependent process, outlasting the freeing of the opiate receptors from agonist must be responsible, since morphine pretreated mice continued to be tolerant after 8 and 16 hours of abrupt withdrawal, although morphine brain levels had declined to such an extent that, by eight hours, specific opiate binding sites were no longer blocked by morphine. This agrees with a report of Schulz and Herz (1976), who found that tolerance to opiate effects in the myenteric plexus-longitudinal muscle of the guinea pig, continued to exist over a period of hours, despite continuous washing to remove opiate agonist from the tissue.

D. Measurement of the apparent affinity of naloxone.

Because previous experiments using drug concentrations to trace receptor binding in vitro and in vivo were unable to uncover changes in the receptor related to tolerance, the following experiments employed a behavioral method.

1. proportionality of morphine brain content with injected dose:

Because the apparent affinity constant is only proportional to the actual affinity constant of the antagonist when the concentrations of drugs in the brain is directly proportional to the dose given (see section III D), it was necessary to demonstrate that the morphine content in the brains of naive and tolerant animals correlated linearly with dose. This was done by injecting a large range of doses of morphine into naive (1 to 20 mg/kg) and tolerant rats (10 to 200 mg/kg) and measuring morphine content in the brains of these animals 45 minutes later. Morphine concentrations at this time after injection, when analgesia was measured, correlated linearly with the dose administered (see also Bläsigg et al., 1978).

2. naloxone antagonism of morphine analgesia in naive and tolerant rats:

The ability of naloxone to antagonize morphine was

estimated by measuring the size of shifts to the right, caused by three doses of the antagonist, of morphine dose-response curves for analgesia.

Because of the size of the variation in the results, a minimum of 60 (and as many as 171) rats were used to measure a single dose-response curve, meaning that an average of about 300 rats were needed to estimate the apparent pA_2 constant of one group.

The results of the naive experiment are represented in Fig. 10a. The morphine dose-response curve in the absence of naloxone had an ED_{50} of about 7.4 mg/kg. Naloxone doses of 0.1, 0.4, 1.6 mg/kg (i.p.) shifted this curve to the right, increasing the ED_{50} to 21.9, 55.3 and 212.7 mg/kg, respectively. The slopes of the curves in the presence of naloxone were parallel to that in the absence of the antagonist, a strong indication that naloxone and morphine compete for the same receptor in a similar manner.

The dose-response curves, in the presence and absence of naloxone, of the tolerant rats are pictured in Fig. 10B,C,D. As in the naive rats, naloxone shifted the morphine dose-response curve, obtained in the absence of the antagonist, parallel to the right. However, the shift was somewhat larger in all of the tolerant rats.

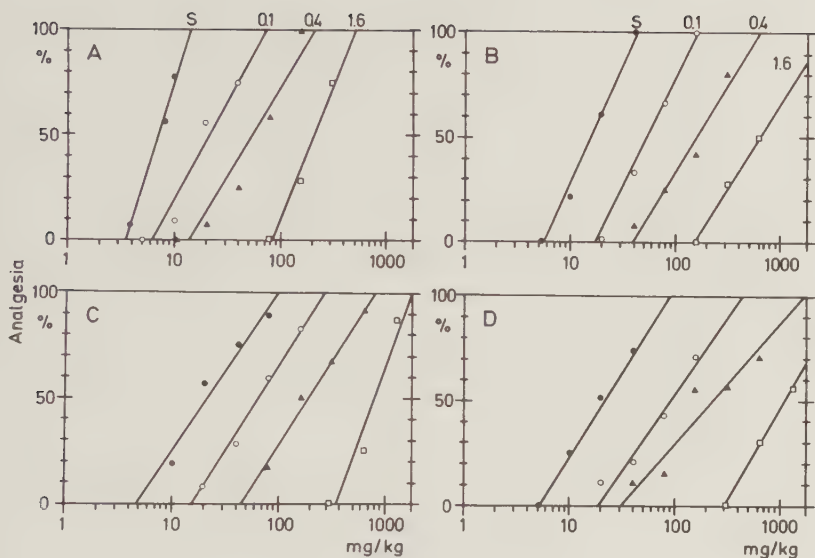


Fig. 10: Morphine dose-response curves for analgesia in the presence and absence of naloxone administration in naive (A) and in various tolerant groups: 1/4 group (B); 2/7 group (C) and 3/7 group (D). Abscissa: dose of morphine (s.c.) injected 45 min. before analgesia test; ordinate: % analgesia. Naloxone or saline injected 20 min. before test. Each point is the mean of 18-36 rats. Dose of naloxone: 0.1 (○), 0.4 (▲) and 16 (◻) mg/kg, i.p., shifted the morphine ED_{50} of the saline-injected controls (●) in each group as follows: Naive from 7.40 to 21.9, 55.3 and 212.7 mg/kg; 1/4 from 15.4 to 53.1, 158.4 and 623.5 mg/kg; 2/7 from 20.87 to 65.65, 187.8 and 783.3 mg/kg; 3/7 from 21.80 to 91.4, 228.7 and 1066.7 mg/kg, respectively.

For example, 1.6 mg/kg naloxone increases the ED_{50} of morphine in the tolerant groups by factors 41, 38 and 49 for the 1/4, 2/7 and 3/7 rats, respectively, while it shifts that of the naive group only by a factor of 29. Thus, antagonistic potency of naloxone appears to be increased in the tolerant rats. The dose-response curves of the tolerant animals also appear somewhat flatter than those of the naive animals. This picture changes, however, if the amounts of chronic morphine (i.e. originating from the implanted pellet (s)) in the brains of the tolerant animals are taken into consideration.

GROUP	CHRONIC "PELLET" MORPHINE IN THE BRAIN (ng/gm brain)	EQUIVALENT DOSE OF MORPHINE (mg/kg s.c.)
1/4 ^Δ	97.5 $\pm$ 21	6.3
2/7	135 $\pm$ 30	8.3
3/7	187.5 $\pm$ 29	11.0

Table 4: Concentration of chronic "pellet" morphine in the brains of tolerant rats implanted with pellets containing morphine and the dose of morphine needed to produce this concentration in naive rat brains. Values are the means of 8-10 animals, with standard deviation. Doses are extrapolated from a dose-concentration plot in tolerant and naive rats (see Bläsigg et al., 1978). ^ΔCode: The first number is the total quantity of pellets implanted, the second the number of days of pretreatment.

The chronic morphine levels in the brains of the tolerant/dependent rats were measured, and the doses of morphine necessary to produce these concentrations calculated from the calibration curve done in naive rats (Table 4). Dose-response curves of tolerant rats were then corrected by adding the dose-equivalent of the morphine concentration in the brain of the tolerant rats to the dose injected. After this procedure, naloxone still shifts the morphine dose-response curve parallel to the right (see Fig. 11C,D,E). However, the magnitude of this shift is diminished to about the same as that seen in the naive group. Thus, the antagonistic potency of naloxone does not seem to be changed in the tolerant animals when the "pellet morphine" is considered.

Because this finding was surprising in view of evidence that morphine pretreatment with as little as one dose increases the potency of naloxone (Tulunay and Takemori, 1974a,b; Frederickson, 1974), naloxone antagonism was tested in rats which had received only a single pretreatment dose of morphine (8 mg/kg s.c., 6 hrs before testing). At the time of testing, no analgesia from the original dosage could be measured; however, a small "acute tolerance" (shifted ED_{50} of morphine from 7.4 to 8.0 mg/kg ; not statistically sig. $p > 0.05$) could be seen. Less than 12 ng free morphine per gram wet

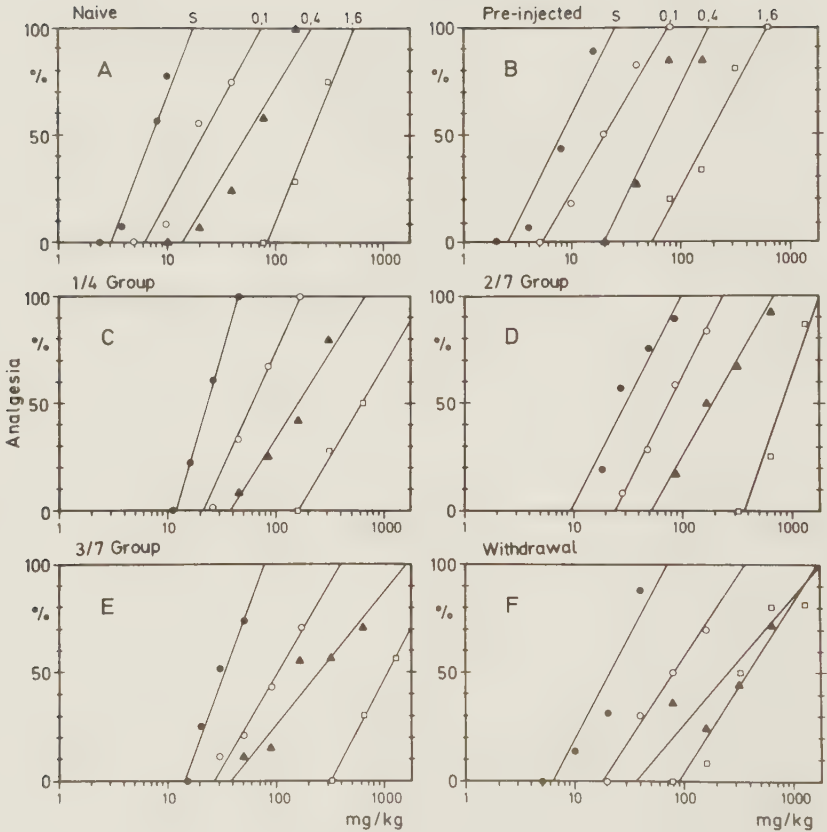


Fig. 11: Morphine dose-response curves for analgesia in the presence and absence of naloxone administration in naive (A) rats; in pre-injected rats (8 mg/kg morphine s.c. 6 hrs before analgesia test) (B); in rats with

various degrees of tolerance/dependence: 1/4 (C), 2/7 (D) and 3/7 (E) groups; and in rats (1/4 group) in abrupt withdrawal (6 hrs after pellet removal) (F). Abscissa: dose of morphine, injected s.c. 45 min before analgesia test; Ordinate: % analgesia. Naloxone or saline injected (i.p.) 20 min before test. Each point is the mean of 18-36 rats. Dose-response curves for tolerant rats were corrected for the "pellet morphine" in the brain (see results). Naloxone, 0.1 (○), 0.4 (▲), 1.6 (□) mg/kg, shifted the morphine ED_{50} of the saline injected controls (●) in each group as follows: naive from 7.40 to 21.9, 55.3, 212.7 mg/kg; pre-injection from 8.0 to 19.9, 58.6, 185.0 mg/kg; 1/4 from 23.1 to 61.0, 166.4, 629.2 mg/kg; 2/7 from 30.5 to 76.3, 195.7, 792.7 mg/kg; 3/7 from 33.4 to 102.6, 245.5, 1077 mg/kg; abrupt withdrawal from 21.5 to 82.5, 260.7, 387.4 mg/kg, respectively.

brain, which is equivalent to a dose of less than 1 mg/kg, is left in the brain after the morphine pre-injection dose at this time. Therefore, the dose-response curves were established on the basis of the morphine test dose alone, i.e. without correction. As seen in Fig. 11B, naloxone produced parallel shifts of the morphine dose-response curve of about the same magnitude, on the log scale, as observed in the naive group.

The potency of naloxone was also explored in rats during abrupt withdrawal (1/4 group), 6 hours after pellet removal (see Fig. 11F). The animals showed signs of withdrawal, such as diarrhea, screaming on

touch and increased aggression. At this time, less than 10 ng of the pellet morphine per gram wet brain weight is left in the brain, making the correction of the dose-response curves unnecessary. The potency of naloxone in the withdrawal group was difficult to evaluate. Although the experiment was repeated three times, a linear relationship between the shift of the morphine dose-response curve and the dose of naloxone given could not be established. This made comparison with the results from the naive group difficult. For example, although the shift of the ED_{50} caused by 0.4 mg/kg naloxone was much larger than that seen in the naive group at this dose, the shift caused by 1.6 mg/kg naloxone was smaller than that of the naive group.

3. evaluation of apparent pA_2 constants for naloxone:

Fig. 12A presents the pA_x plots for the measured results (uncorrected) from the tolerant/dependent animals in comparison with those of the naive group. All of the pA_x lines from the tolerant/dependent animals are shifted to the right of the naive line, although the slopes (-1) are about the same as that of the naive group. Thus the pA_2 values of the tolerant animals, 6.94, 6.84, and 7.03 for the 1/4, 2/7 and 3/7 groups, respectively, are all somewhat larger than that of the naive animals, 6.83, making it appear

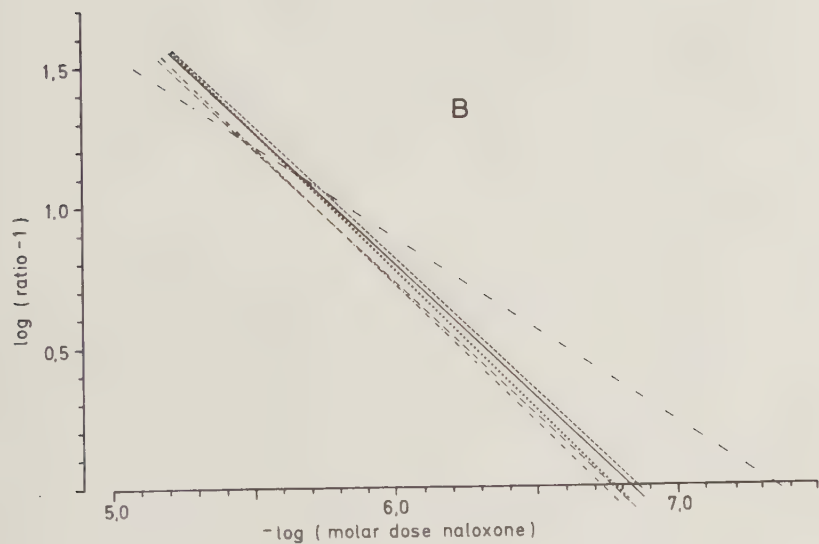
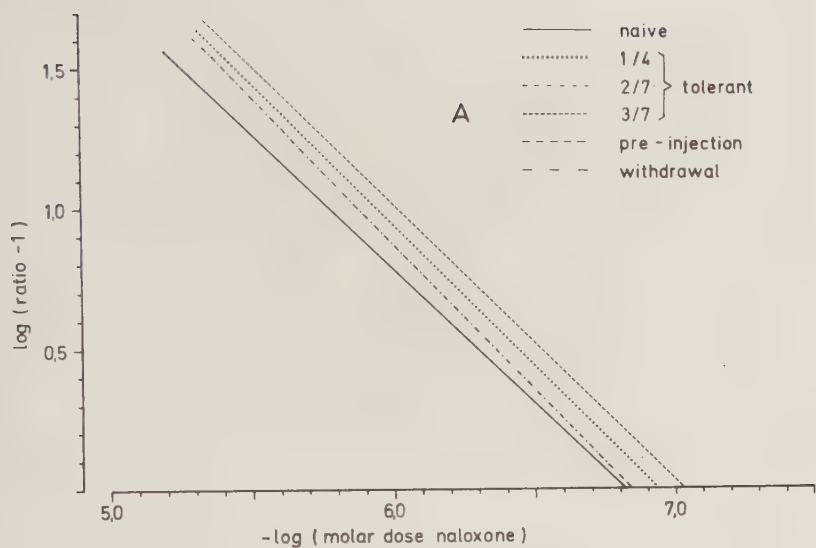


Fig. 12

Fig. 12: (A): pA_2 plots of naive rats (—); of the rats with various degrees of tolerance: 1/4 group (.....); 2/7 group (-----); 3/7 group (-----), taken from data of Fig. 10, where dose-response curves were not corrected for chronic "pre-treatment" morphine in the brains. (B): pA_2 plots of naive (—), same as above, and of pre-injected rats (8 mg/kg morphine s.c. 6 hours before analgesia test) (— — —); of rats with various degrees of tolerance: 1/4 group (.....); 2/7 group (---) and 3/7 group (-----), taken from data of Fig. 11, where dose-response curves were corrected for chronic "pre-treatment" morphine in the brain; and of a group in abrupt withdrawal, 6 hrs after pellet removal (— · — · —).

as if the apparent affinity constants had increased by 29%, 2% and 58% for the respective tolerance groups. This impression is changed, however, if the pA_x lines are calculated from the corrected dose-response curves.

Fig. 12B presents the pA_x plots for each group when the corrected results are used. The pA_x lines of the tolerant animals now appear clustered close to that of the naive group and the slopes remain close to -1. The pA_x curve of the pre-injected group is also close to that of the naive. There is no significant difference ($p < 0.05$) between the apparent affinity constant found for this group and that of the tolerant or naive groups. Neither is there any correlation

between the size of these values and the degree of tolerance. The average of all constants, including the naive group, is around 6.8. On the other hand, the pa_2 value of the withdrawal group (7.36) is significantly different from that of the naive group ($p < 0.05$). This result can, however, not be simply interpreted as a change in the apparent affinity constant because the slope of the pa_x curve is not -1, as is predicted by the simple law of mass action (see section III D).

4. discussion:

Naloxone appeared to be somewhat more effective in antagonizing morphine analgesia in tolerant than in naive animals; however, this increase disappeared when the dose-response curves of the tolerant animals were corrected for the amount of chronic morphine (originating from the pretreatment) in the brains of these animals. The ability of naloxone to antagonize morphine analgesia in animals pre-injected with morphine was also about the same as that in the naive group. In contrast, it was impossible to compare the potency of naloxone in a group of rats in withdrawal with that in the naive, because the antagonistic effect of naloxone was not linearly proportional to the dose of naloxone given in this group. The apparent pa_2 values of th

tolerant/dependent groups were consistently larger in the tolerant groups than in the naive animals, making it appear as if the apparent affinity constant had increased during the development of tolerance. This is not surprising, since the simple theory of mass action predicts that the presence of agonist in the brains of animals would artificially inflate the pA_2 value (see section III D). When the dose-response curves of the tolerant groups are corrected for the "pellet morphine", this increase disappears. The apparent pA_2 value of the acutely pre-injected group was also very close to that of the naive animals. The slopes of the pA_x curves of all of these groups were close to that predicted by theory, -1. The pA_x line of the group of animals in withdrawal was flatter, with a slope of only -0.65. Therefore, although the apparent affinity constant of this group was significantly different from that of the naive, this could not be interpreted as a change in the apparent affinity constant of this group (see section III D). Thus, these experiments provide no evidence that a change in the affinity constant of the antagonist takes place during the development of tolerance/dependence.

The apparent pA_2 value in naive rats was 6.83, which is equivalent to an apparent affinity constant of naloxone of 6.76×10^6 liter/mole. This value is very

similar to that reported by Takemori et al. (1973) and by Smith (1976) using the apparent pA_2 method in mice. These values are smaller than the affinity constant measured in vitro, as reported in this thesis (see section V A). Some difference may be expected, however, partly because the apparent affinity values are based on doses administered instead of on actual molar concentrations in the brain and partly because of the possible effects produced by the preparation of the receptor for in vitro analysis.

The dose-response curves of the tolerant (pellet implanted) animals were corrected by adding the dose of morphine needed, in naive animals, to produce the concentration of the drug measured in the brains of the pelleted animals to the dose of morphine injected. Two assumptions underly this procedure: 1) that a dose of morphine administered to a naive animal is absorbed in the same way as the same dose given to a tolerant animal and 2) that a more-or-less constant, chronic level of the agonist interacts with the morphine receptor in the same way as an acute level of the drug. The first assumption is justified by the observation that the graph of dose against brain concentration in naive rats is identical with the same graph for tolerant animals, when the dose-equivalent of the initial amount of morphine in the brain, due

to pretreatment, is added to the dose administered. The second assumption can be justified because analgesia was tested 45 minutes after morphine injections, at a time when analgesia had been at a plateau for about 15 minutes, an indication that morphine had reached equilibrium with the receptors. Another important consideration is, of course, whether or not the pellet morphine is as free to interact with receptors as the injected drug, i.e. has not become more tightly bound to receptors or tissue as compared to acute doses of the drug. As seen in the last experiment, this is not the case in mice. In rats, the seriousness of this possibility can be best judged by looking at the decay of morphine brain concentrations in tolerant and naive animals. Recent experiments (Bläsigg et al., 1978) indicate that the decay of morphine concentration from the brain of tolerant rats after pellet removal is very similar to that seen in naive rats following an acute injection (Misra et al., 1971). If morphine was bound to any great extent in a different way in tolerant animals, the decay curves would be expected to be different.

It is important to discuss the claim of Takemori (Tulunay and Takemori, 1974a,b) that morphine pretreatment according to various schedules may increase the potency of naloxone and the apparent affinity of

the antagonist for the opiate receptor. In one of these experiments, the apparent pA_2 value was calculated in mice in abrupt withdrawal (Tulunay and Takemori, 1974b). When the results are closely inspected, it is evident that the slope of the pA_x curve for these animals is -0.64; that is, almost exactly the same as that found for rats in abrupt withdrawal reported on in this experiment. It is possible that the same factors which hindered the measurements of the apparent affinity constant in rats were also at work in Takemori's mice. Furthermore, some of the other data presented by the Takemori group are inconsistent with the interpretation of the authors. For example, they claim that a pretreatment of mice with 3 x 30 mg/kg morphine causes the development of low-level tolerance and a 70% increase in naloxone potency. In contrast, a comparison of the Takemori data from the naive and tolerant mice by computing the factors by which the morphine dose-response curves are shifted in the different groups, reveals that naloxone had perhaps become slightly less effective in the tolerant mice (Tulunay and Takemori, 1974b). The strength of the conclusions of these authors are also reduced because they are partially based on tests with only a single dose of antagonist (Tulunay and Takemori, 1974a). The above authors also fail to evaluate the possibility that morphine may remain in the brain of the mice following

pretreatment injection, given only a few hours before tests. A similar oversight was made by Wong and Bentley (1978) who assumed that three hours after a pretreatment dose of morphine (2 mg/kg s.c.), no residual morphine would be in the mouse brain because no analgesia effect was observed. However, previous experiments (Dum et al., 1977) show that the half-life of morphine in the brain is about 2.5 hours. Considering that time is needed for morphine to enter the brain after injection, the equivalent of 1 mg/kg was probably in the brains of the pretreated mice. This dose is about equivalent to the ED_{50} morphine in the Wong and Bentley experiment. Since these authors failed to measure analgesia in this group, acute tolerance had undoubtedly developed. This would explain why the potency of naloxone appeared increased in these experiments.

A report of Smith (1976) that a single pretreatment of mice with an agonist causes a change in the PA_2 of naloxone to antagonize opiate analgesia, but not to antagonize the catacractogenic effect is also open to question, since the mice had developed tolerance to both effects. To explain this paradox, Smith postulates that two receptor systems are behind the two responses. However, this contention is difficult to reconcile with the fact that the author finds the

pA_2 of naloxone for these responses to be the same in naive animals, implying that the effects are produced by the same receptor types. It is difficult to propose an explanation for these inconsistencies, but they offer little support to the suggestion that tolerance/dependence takes place at the opiate receptor binding site.

E. Changes in the dose-response curve of morphine during the development of tolerance and dependence considered in the light of receptor theory.

The previous experiment was unable to find a change in the effectiveness of a morphine antagonist, naloxone. Therefore, the following behavioral experiments were performed to distinguish if changes in the dose-response curve of the agonist, morphine, could be related to changes in receptor characteristics in tolerant/dependent animals.

1. morphine in the brains of the tolerant rats:

Table 5 lists the amount of morphine in the brains of tolerant animals, accompanied by the morphine dose-equivalent of this amount in mg/kg. This dosage was added to the dosages actually administered in order that a correct estimate of the dose-response curves for these animals could be made. This was necessary since the chronic morphine levels would otherwise cause an apparent flattening of the curves (see section III E).

GROUP	CHRONIC "PELLET" MORPHINE IN BRAIN (ng/gm brain)	EQUIVALENT DOSE OF MORPHINE (mg/kg s.c.)
1/4 ^Δ	97.5 ± 21	6.3
3/7	187.5 ± 29	11.0
6/10	267 ± 38	15.0
13/10	780 ± 46	39.0
24/10	1142 ± 276	54.0

Table 5: Concentration of chronic "pellet" morphine in the brains of tolerant rats implanted with pellets containing morphine and the dose of morphine needed to produce this concentration in naive rats. Values the mean of 8-10 animals, with standard deviation. Δ Code: The first number is the total quantity of pellets implanted, second the number of days of pretreatment.

2. morphine dose-response curves in naive and tolerant rats:

Fig. 13 shows the dose-response curve of morphine in naive and in five groups of rats with increasing degrees of tolerance. The ED_{50} morphine of the naive group is about 7.4. In the two groups with the lowest degrees of tolerance, the 1/4 and the 3/7 groups, the ED_{50} 's are increased to 13 mg/kg and 28 mg/kg, respectively. The slopes of the dose-response curves are not different from that of the naive ($p > 0.05$). However, when tolerance is increased beyond this level, the dose-response curves become flatter (significant $p < 0.05$), and begin to reach a plateau at about 300 mg/kg. As a result, the maximum analgesia which can be induced drops. In the most tolerant group, the highest level of analgesia lies below 20%. The plateauing is observed to occur within approximately the same dose-range in all three of the highly tolerant groups.

3. the in vivo displacement from the brain of rats of a trace concentration of 3H -etorphine by increasing doses of unlabelled morphine:

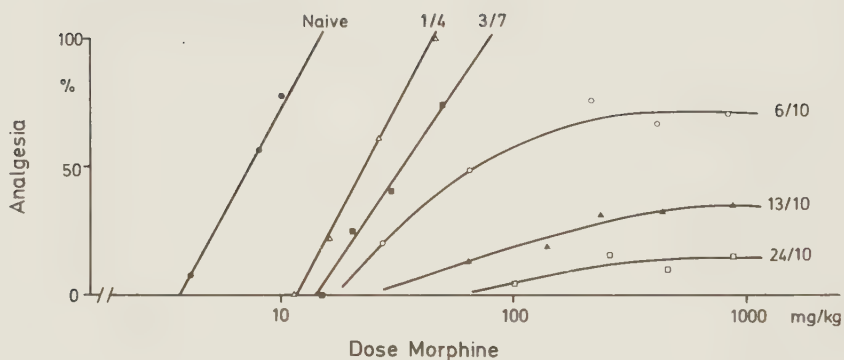


Fig. 13: Morphine dose-response curves for analgesia in naive rats (●) and in groups with increasing degrees of tolerance: 1/4 group (▲); 3/7 group (■); 6/10 group (○); 13/10 group (△); and 24/10 group (□).
Abcissa: dose of morphine (s.c.) injected 45 min. before analgesia test; Ordinate: % analgesia. Each point is the mean of 18-36 rats.

In order to relate the changes in the morphine dose-response curves, during the development of tolerance/dependence, to receptor occupation, the in vivo displacement of a small concentration of labelled opiate agonist from the brain by increasing doses of morphine was measured. This was done in tolerant rats (6/10 group) so that the rats would survive the large doses of morphine needed for this experiment. It was, therefore, necessary to correct the displacement curve for the pretreatment

morphine in the brain by adding the dose-equivalent of the chronic morphine concentration to the dose actually given, i.e. in the same manner in which the analgesia dose-response curves were corrected. As shown in fig. 14, doses of morphine between 5 and 1000 mg/kg logarithmically reduce the ratio of the concentration of labelled substance in the brain (without cerebellum) to concentration in the cerebellum from 1.9 down to 1.1. The ratio reaches a minimum plateau when a dose of around 300 mg/kg morphine is given, suggesting that this dose produces a concentration in the brain high enough to saturate the opiate high affinity binding sites.

4. discussion:

The dose-response curve of morphine is shifted parallel to the right of the naive rats in the groups with lower degrees of tolerance. Such a shift would be expected if a decrease in receptor affinity for the agonist had taken place. However, a reduction in the efficacy of the drug would also cause a parallel shift in the dose-response curve if the maximum response measured was not related to the occupation of all receptors. In this case, a flattening of the curve would first become visible when the reduction in efficacy was so large that receptor saturation was necessary to produce the maximum response. Such a flattening of the curve is indeed

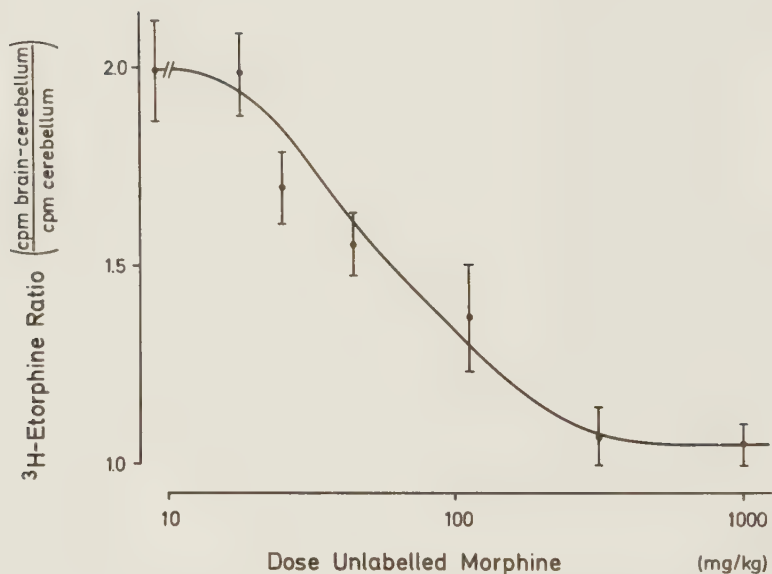


Fig. 14: The reduction of the ratio of the concentration of ³H-etorphine in the brain (without cerebellum) to concentration in the cerebellum (brain/cerebellum ratio) by increasing doses of unlabelled morphine. ³H-etorphine injected i.v. 20 min. and unlabelled morphine s.c. 45 min. before sacrifice; 12 rats/point; standard error illustrated. Rats are tolerant (6/10 group) so that the high doses of morphine are tolerated. Curve corrected for chronic levels of morphine in the brains of the rats.

observed in the highest tolerance groups. Further evidence that the opiate receptors are not saturated at the maximum response measured in the naive and lower tolerance groups is presented by the in vivo

displacement curve, which indicates that a dose of 300 mg/kg is necessary to cause receptor saturation. The maximum response in the naive and lower two tolerance groups occurs, in contrast, below this dose. In the highest tolerance groups, the dose-response curves show no further increase in response beyond this dose and the maximum response decreases. Since a change in affinity alone could not cause such a flattening, the simplest explanation for the curves is that a change in efficacy has taken place. Apparently, either the upper part of the S-shaped dose-response curve, reflecting the gradual occupation and saturation of the receptor population, is not recorded by the analgesic test procedure in the naive rats and in the lower tolerance groups, or maximum analgesia is produced by occupation of only a portion of the total number of receptors, i.e. spare receptors exist. Therefore, a change in efficacy is able to mimic a change in affinity. These conclusions are supported by recent investigations into the ability of naloxone to precipitate withdrawal in rats with increasing degrees of tolerance (Bläsigt et al., 1978), which showed a progressive change in the quality of the response of the receptors to occupation by the antagonist during the development of tolerance/dependence. The findings of Way et al. (1969), that naloxone is able to precipitate withdrawal at lower doses as tolerance/dependence increases,

may also be best explained in this way. The suggestion that a change in the opiate receptor binding site occurs during the development of tolerance/dependence can not be supported.

VI. CONCLUSIONS

None of the experiments performed were able to find a change in the interaction of opiates with their receptors related to tolerance/dependence, although five series of experiments were performed using different techniques, which investigated the question from different angles.

The first group of experiments measured high affinity opiate-specific binding of a highly labelled opiate agonist and antagonist in rat brain homogenate. By measuring a displacement of this binding with the respective unlabelled ligands, estimates of the affinity and number of binding sites could be found for the different substances using Scatchard plots. Morphine was removed from the brain tissue of the pretreated animals by repeated washing. With this approach, no change in the opiate receptor characteristics, for agonist or antagonist, in the tolerant/dependent animals, could be observed. Nevertheless, these experiments could not exclude the possibility that receptor changes take place which depend upon the support of the in vivo environment of the receptor. Therefore, experiments were also performed in living animals.

The first in vivo experiments compared the effect of a dose of antagonist on concentrations of morphine in the brains of tolerant mice with that in the brains of

naive mice. Other investigators had reported that a displacement in tolerant mice (Shen and Way 1975a, b; Way et al., 1976), but not in naive mice (Way, personal communication) takes place and suggested that this implied a change in the affinity of the brain for the agonist or antagonist. Nevertheless, with very similar treatment schedules, experimental animals, and a very nearly identical assay method, no displacement of morphine from the brains of either naive or tolerant animals with the antagonist could be found. Thus, the findings of the Way group could not be supported. It appears that, in order to study opiate receptor interactions in living animals, a more sensitive method is needed.

The next series of experiments measured brain concentrations, in living animals, of drugs which, in contrast to morphine, have relatively high affinities for the opiate receptor and which, therefore, in low concentrations, have a larger proportion of their molecules associated with opiate receptors. Consequently, the levels of these substances in the brain decrease when displaced from specific opiate binding sites by an antagonist. Unspecific pharmacokinetic factors are controlled for with the concentration of labelled drugs in the cerebellum, which largely lacks specific opiate receptors (see section III C). Thus, with this method, a comparison of opiate receptor interactions, in living naive and tolerant/dependent animals was possible. No evidence of a change in receptor

number was found. Some differences which did exist between the displacement in naive and tolerant mice of labelled agonists and antagonists, were of the same nature and magnitude predicted to result from competition for the receptors of the morphine in the brain of the pretreated animals and disappeared as morphine left the brain during abrupt withdrawal, although tolerance was high. Furthermore, displacement in the tolerant animals failed to show any more changes beyond eight hours after the beginning of abrupt withdrawal, after morphine levels had dropped below 18 ng/g wet brain, although tolerance was declining at this time. From this it could also be concluded that, although pretreatment morphine competed for the opiate receptor binding sites in the tolerant group not in withdrawal, this morphine was not directly responsible for the maintenance of tolerance. Tolerance must, instead, be a time-dependent process outlasting the freeing of opiate receptors from agonist. No evidence was found of a change in opiate receptor characteristics in the tolerant/dependent groups.

In order to investigate opiate receptor interaction from still another viewpoint, the next experiment applied a behavioral method which measured the ability of an opiate antagonist to antagonize morphine analgesia in

rats, so that the apparent pA_2 value could be derived. Since this value should be proportional to the affinity constant of the antagonist, an alteration in the affinity of the receptor for the antagonist in the tolerant/dependent animals should be reflected in a change in the apparent pA_2 value. The dose-response curves of the animals with pretreatment morphine in the brains were adjusted to compensate for the competitive action of this drug. With this approach, no indication of a change in antagonist potency or in the apparent pA_2 value of the antagonist in different groups of tolerant rats or in rats with low-level acute tolerance could be found. Measurements made in rats during abrupt withdrawal could not be interpreted in terms of the apparent pA_2 value of the antagonist because the pA_x curve did not have a slope of -1, which is required for application of the pA_2 method. Therefore, using this behavioral method, no change in receptor affinity for the antagonist could be found in the tolerant animals.

Another series of experiments were performed in order to apply a behavioral test to the possibility that the receptor affinity for the agonist might be changed in tolerant animals. This was done by comparing dose-response curves for morphine analgesia in naive and increasingly tolerant rats. As in the last experiment, the dose-response curves were adjusted to compensate for the

competitive influence of morphine in the pretreated animals. When this was done, a flattening of the curves, with a reduction in maximum effect, indicative of a reduction in efficacy, was seen in the three highest tolerance groups. In contrast, parallel shifts of the dose-response curves of the two lower tolerance groups were observed. However, in vivo displacement studies revealed that the maximum response measured in these groups did not correspond to receptor saturation. Therefore, a reduction in efficacy would also explain the parallel shift in these curves, since the alteration in the maximum response normally expected to accompany a reduction in efficacy would not be visible in this case. Thus, the simplest explanation is that the efficacy, or reaction to opiate receptor occupation, is diminished in both high and low tolerance groups. It is not necessary to propose a change in receptor characteristics.

The various experiments presented in this paper support and supplement each other. For example, the in vitro analysis of receptor binding is a robust approach, because, with it, high affinity opiate-specific binding can be easily distinguished from free and low affinity, unspecifically bound opiate. Since the concentration of opiate in the brain homogenate is also known, receptor number and affinity can be directly calculated. In contrast, in vivo displacement depends on the use of high affinity opiates

to make the receptor binding visible and pharmacokinetic factors must be considered. Nevertheless, this approach probes receptor characteristics under physiological conditions. It allows correlation of receptor binding with receptor effect, so that, in the last experiment, it was possible to see if receptor saturation correlated with the maximum effect and, in another, to judge if agonist occupation of the opiate receptor binding sites was directly necessary for the maintenance of tolerance. Behavioral methods, though somewhat indirect, also add their own unique form of information. With the pA_2 method, it was possible to contrast the shift caused in the dose-response curve, due to the development of tolerance to the agonist, with the lack of change in potency of the antagonist, thus giving insight into opiate interactions with functioning receptors. Similarly, the last series of experiments, by analysing the functional relationship between drug dose, receptor occupation and response, was able to differentiate a change in reaction of receptor occupation from the interaction of the agonist with the receptor binding site. Thus, a composite picture can be made, which shows no change in opiate receptor interaction in the tolerant/dependent animals, but rather the impression of a time-dependent process operating beyond the level of the opiate binding site, causing an alteration in the response of the organism to opiate receptor occupancy.

It is important to note that the validity of each experimental series rests upon the self-consistent application of one approach to all experimental groups. Thus, although each method has a particular bias, this should not be relevant to a comparison of results between the different groups. In each approach it is necessary to eliminate any variables, such as unspecific differences in pharmacokinetics, behavioral changes or pretreatment drug in the brains of the tolerant/dependent animals, which might otherwise tend to cause the appearance of differences between groups, particularly in vivo, where no change in receptor binding characteristics exists. Failure to attend to some of these variables may explain why several researchers have found evidence for changes at the receptor level during the development of tolerance and dependence (see individual discussions for details). In all of the experiments reported here, great emphasis was placed upon controlling for such interfering factors.

It is interesting to compare the parameters of the opiate receptor binding sites as measured by the various techniques. Two experiments measured the number of opiate receptor binding sites, the first experiment, done in rat brain homogenate and the third experiment, which traced the in vivo displacement of radioactive drugs from the mouse brain with unlabelled ligand. Both of

these experiments, which had totally different methodology, found a receptor number of about 20 pmoles/gm wet brain. This estimate is in line with the findings of other investigators (see Höllt and Wüster, 1978). The affinity of the opiate receptor for naloxone was also estimated in the two above experiments and the apparent affinity for naloxone was estimated in the fourth experiment, using a behavioral approach. The various values found were 9.86×10^8 , 5.65×10^7 and 6.76×10^6 liters/mole measured with the in vitro, the in vivo displacement and the behavioral methods, respectively. Since the behavioral method actually measures the apparent affinity constant which is based on the dose of drug, rather than the affinity constant itself, it is not surprising that the estimate made is the lowest. There is probably less drug in the brain at the time of testing than assumed from the dose. The in vivo displacement procedure may also have some bias because the approximation of the free drug concentration at the receptor, based on the concentration in the cerebellum, may be slightly overestimated (see section III C for details). It is therefore tempting to accept the in vitro results as providing the definitive estimate of the affinity constant. However, the opiate receptor binding site is unstable under in vitro conditions (Pasternak and Snyder, 1973) and is ion sensitive (Simon, 1975a,b; Pert and Snyder, 1974). Therefore, the normal characteristics of the binding site could also be

altered in these experiments. As a result, the in vivo results may be more reliable and best reflect the characteristics of the functioning receptor. This contention is supported by experiments which are able to directly correlate in vivo displacement data with the pharmacological effects in the living animal (Hölldt and Herz, 1978).

In the introduction to this paper, it was pointed out that the mechanism of tolerance must be looked for within the nervous system itself. Since the results of these experiments consistently agree that a change in opiate receptor interaction is not responsible for tolerance, the mechanism must be such that either the reaction of the tissue or cell to the drug is changed, or compensatory effects are produced which exactly cancel opiate action. From the studies of tolerance presented in this paper, it is not possible to distinguish between these two possibilities. However, the linkage between opiate tolerance and dependence suggests that one mechanism lies behind both, which reestablishes normalcy to animals under the influence of opiates, and, in so doing, primes the organism for withdrawal. From this it follows that a compensatory process may be operating. This suggestion is also supported by data indicating changed sensitivity of the peripheral and central nervous system to nervous transmitters in tolerant/dependent

animals (Schulz, 1978; Satoh et al., 1976a; Carlson, 1977). Since such changes in sensitivity towards neural transmitters, as well as to opiate agonists and antagonists, could be observed even at the single cell level (Satoh et al., 1976a,b), attention should be directed towards understanding the intracellular, biochemical counterpart of these changes. The reward should be a better understanding of the way in which sensitivity to endogenous substances is regulated in the nervous system.

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